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"REGULACIÓN DE LA ESPERMATOGÉNESIS Y RESPUESTA INMUNITARIA POR MOLÉCULAS DE LA MATRIZ EXTRACELULAR EN PECES TELEÓSTEOS"

Memoria que presenta

Dña. Patricia Castillo Briceño

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Abreviaturas

Abs absorbancia

Actb beta actina

AGs granulocitos acidófilos

ANOVA análisis de varianza

BMP proteína morfogénica ósea

BSA albúmina de suero bovino

CCL4 quimioquina (motivo C–C) ligando 4

cDNA copia de ácido desoxirribonucleico desde mRNA

COL colágeno

COX ciclooxigenasa

Cq ciclo de cuantificación (en PCR a tiempo real)

DAMPs patrones moleculares asociados a daño

DDRs receptores dominio discoidina

DNase I desoxirribonucleasa I

dph días después de la eclosión

E2 17β-estradiol

Ech equistatina

ECM matriz extracelular

EDTA ácido etilendiaminotetraacético

EE2 17α-etinil-estradiol

EEC células endoteliales

ELISA ensayo inmuno-enzimático

End endostatina

FBS	suero bovino fetal
h	horas
HK	riñón cefálico
IL	interleuquina
ITG	integrina
LAIRs	receptores tipo inmunoglobulina asociados a leucocitos
LPS	lipopolisacárido
MACS	aislamiento de células magnéticamente activadas
MCs	macrófagos
M-CSF	factor estimulante de colonias de macrófagos
min	minutos
MMP	metaloproteasa de la matriz
NO	óxido nítrico
OSN	osteonectina
p.e.	por ejemplo
PAMPs	patrones moleculares asociados a patógenos
PBS	tampón fosfato salino
PCR	reacción en cadena de la polimerasa
PMA	forbol miristato acetato
RC	ciclo reproductivo
mRNA	ácido ribonucleico mensajero
RNIs	intermediarios reactivos de nitrógeno
ROIs	intermediarios reactivos de oxígeno
rps18	proteína ribosomal S18

RT-qPCR PCR de transcripción reversa a tiempo real

TIMP inhibidor tisular de MMPs

TGF factor transformante del crecimiento

TNF factor de necrosis tumoral

UV luz ultravioleta

VaDNA DNA genómico de *Vibrio anguillarum*

Listado de especies

Nombre científico	Nombre común / Common name
Bos taurus (Linnaeus, 1758)	ganado bovino / bovine cattle
Ciona intestinalis (Linnaeus, 1767)	ascidia amarilla / transparent sea squirt
Cyprinus carpio (Linnaeus, 1758)	carpa común / common carp
Danio rerio (Hamilton, 1822)	pez cebra / zebrafish
Gadus morhua (Linnaeus, 1758)	bacalao del Atlántico / Atlantic cod
Homo sapiens (Linnaeus, 1758)	humano / human
Ictalurus punctatus (Rafinesque 1818)	bagre de canal / channel catfish
Mus musculus (Linnaeus, 1758)	ratón / mouse
Oncorhynchus mykiss (Walbaum, 1792)	trucha arcoiris / rainbow trout
Oryzias latipes (Temminck & Schlegel, 1846)	pez del arroz / medaka fish
Paralichthys olivaceus (Temminck & Schlegel, 1846)	lenguado del Pacífico / Japanese flounder
Rattus rattus (Linnaeus, 1758)	rata / rat
Salmo salar (Linnaeus, 1758)	salmón / Atlantic salmon
Sparus aurata (Linnaeus, 1758)	dorada / gilthead seabream
Synbranchus marmoratus (Bloch, 1795)	anguila de lodo / marbled swamp eel
Takifugu rubripes (Temminck & Schlegel, 1850)	pez globo ocelado / japanese pufferfish
Tetraodon nigroviridis (Procé, 1822)	pez globo de agua dulce / spotted green pufferfish

Nombre científico	Nombre común / Common name
Escherichia coli (Migula, 1895)	
Vibrio anguillarum (Bergeman, 1909)	

RESUMEN

Durante el desarrollo de la presente tesis doctoral se ha estudiado la implicación de diversas moléculas relacionadas con la matriz extracelular (ECM) en la respuesta inmunitaria y en la espermatogénesis de la dorada. Esta especie ha sido ampliamente utilizada en nuestro grupo de investigación, como modelo experimental, debido a sus características biológicas de reproducción estacional, involución testicular y reversión sexual, muy útiles en el estudio de las interacciones entre los sistemas inmunitario y reproductor.

El trabajo se realizó en cuatro etapas, a partir de las interrogantes generadas de la investigación previa en el grupo. Así, la etapa inicial abordó el estudio de la capacidad del colágeno (COL) para modular la actividad de los fagocitos profesionales de la dorada, donde demostramos que ésta molécula es capaz de actuar como una señal de daño (DAMP) que desencadena una respuesta específica, presentando un perfil diferente al de la respuesta establecida ante infecciones o moléculas derivadas de patógenos (PAMPs) en peces. En la siguiente etapa estudiamos las correlaciones existentes entre el perfil de expresión de metaloproteasas de la matriz (MMPs), sus inhibidores tisulares (TIMPs), COL e integrinas (ITGs) como moléculas relacionadas con la ECM. Estas moléculas mostraron estar altamente correlacionadas entre sí bajo condiciones fisiológicas normales y durante el desarrollo, siendo dichas correlaciones moduladas en granulocitos acidófilos durante la respuesta inflamatoria de la dorada ante DAMPs y PAMPs. A continuación, en una aproximación a los procesos iniciales de reparación tisular e inflamación, se estudió la afinidad y el efecto regulador de secuencias específicas y péptidos sintéticos derivados de COL en la actividad celular de fibroblastos de dorada. Estos resultados y los previamente obtenidos, mostraron que tanto el COL nativo, como los fragmentos y los péptidos derivados de COL presentes

en suspensión modulan la respuesta inmunitaria innata y los procesos de inflamación inicial en fagocitos profesionales y fibroblastos de dorada. Finalmente, encontramos que en dorada además de la ITG beta 1a (ITGB1a) que se expresa constitutivamente en todos los tejidos, existe otra isoforma de ITGB1 (ITGB1b) que se expresa principalmente en testículo, donde presenta un incremento drástico y específico durante la espermatogénesis.

Estos resultados convergen con los hallazgos actuales en otros modelos animales que postulan el ambiente de la ECM como un entorno complejo, altamente coordinado y de funcionalidad diversa, donde sus componentes pueden tener un papel como facilitadores en la transmisión de señales o actuar por sí mismos como moléculas señalizadoras o estímulos para la activación celular.

ABSTRACT

The present thesis involved the study of diverse extracelullar matrix (ECM) molecules during the immune response and spermatogenesis in the gilthead seabream. These species has been widely used in our lab as a model due to its biological properties, such as seasonal breeding, testicular involution and sexual reversion, highly useful for the study of interactions between the immune and reproductive systems.

This work was performed in four phases, starting with the study of collagen (COL), as a molecule able to activate professional phagocitic cells. We demonstrate that COL can perform as a damage signal (DAMP) that triggers a specific response with a profile different from the generated by infection of pathogen associated molecular patterns (PAMPs). In the next step, we characterized the correlations between ECM related molecules such as matrix metalloproteases (MMPs), their tissular inhibitors (TIMPs), COL and integrins (ITGs), which were highly correlated during development and in physiological conditions. However, those correlations were modulated in acidophilic granulocytes exposed to DAMP or PAMP stimulation. Subsequently, in an approach to study early wound healing and inflammation processes, we assessed the affinity and regulatory effect of specific motifs and peptides derived of COL on the cellular activity of gilthead seabream fibroblasts. These results, and the previously obtained, showed that native COL as well as its fragments and the synthetic derived peptides in suspension were able to modulate differentially the early innate immune response and inflammation processes in fish professional phagocytes and fibroblasts. Finally, we found that in addition to the ubiquitously expressed ITG beta 1a (ITGB1a) in gilthead seabream, there is another ITGB1 isoform (ITGB1b) which es mainly expressed in testes, whose levels of expression are drastically and specifically increased during spermatogenesis.

These results concurs with the actual findings in other animal models which states that the ECM is a complex environment, highly coordinated and with diverse functionality, where ECM components can facilitate the transmission of molecular signals, or perform as signals by itself, acting as a cell activation stimuli.

ÍNDICE DE CONTENIDOS

CAPÍTULO I: RESUMEN EN CASTELLANO

1 INTRODUCCIÓN A LA MATRIZ EXTRACELULAR

1.1 La Matriz Extracelular

Actualmente la matriz extracelular (ECM) es ampliamente reconocida por su funcionalidad como soporte estructural de órganos y tejidos, como parte de la membrana basal y como sustrato para la migración de células individuales [Hynes 2009]. La composición y organización de las moléculas presentes en el espacio extracelular varían durante el desarrollo, empezando con la fertilización [Rozario y DeSimone 2009]. Además se conoce que las propiedades estructurales distintivas de órganos y tejidos son determinadas principalmente por la ECM y las células que la producen [Tsang et al. 2009].

En un principio, el estudio de la ECM se centró únicamente en su papel como soporte intercelular y tisular, pero a partir del descubrimiento de las integrinas (ITGs) y otros receptores de la ECM en los años 80, se despertó un mayor interés en la ECM como un ambiente complejo y clave para el estudio de diversos procesos celulares y su señalización [Huxley-Jones et al. 2009, Rozario y DeSimone 2009]. Es así que el ensamblaje de componentes de la ECM en superestructuras tales como fibras de colágeno (COL), fibras elásticas y microfibras, hace posible la interacción con varias otras proteínas de la ECM [Tsang et al. 2009], las cuáles a su vez participan en la transducción de señales que regulan el crecimiento, diferenciación, polarización, migración, proliferación y muerte celulares [Hynes 1996, Hynes 2009, Tsang et al. 2009].

El funcionamiento y expresión adecuados de los genes que controlan las interacciones ECM-célula y célula-célula proporcionan el entorno necesario para el normal funcionamiento multicelular de los tejidos [Werb 1997]. En modelos de animales géneticamente modificados, la pérdida o alteración en la expresión de proteínas y receptores citoplásmicos de la ECM, metaloproteasas de la matriz (MMPs) y los inhibidores tisulares de MMPs (TIMPs) generan fenotipos con deficiencias

estructurales, fisiológicas y de desarrollo [Hynes 1996, Lukashev y Werb 1998], sin embargo en algunos casos sus funciones son sustituidas total o parcialmente por genes que codifican moléculas relacionadas [Hynes 1996, Zweers et al. 2007].

De esta manera la participación de la ECM en procesos de adhesión y señalización celular, así como sus propiedades mecánicas, sugieren que las proteínas y señales de la ECM son, al menos, tan importantes para el control de los procesos celulares como las señales solubles ó incluso más que ellas [Hynes 2009].

1.1.1 Colágeno

Los diferentes tipos de COL forman una familia de proteínas compleja desde el punto de vista estructural y del desarrollo que constituyen el componente mayoritario de la ECM en los metazoos [Kramer 1994], para los cuáles ha sido de gran relevancia durante su evolución [Heino et al. 2009]. Asimismo, los diferentes tipos de COL desempeñan un papel predominante en el mantenimiento de la estructura tisular [Myllyharju y Kivirikko 2004, Canty y Kadler 2005], como queda demostrado por el amplio espectro de enfermedades causadas por mutaciones en los genes que los codifican [Myllyharju y Kivirikko 2004, Wenstrup et al. 2004, Wess 2005].

Actualmente se ha identificado alrededor de 30 tipos de COL, de los cuáles siete son fibrilares, es decir que pueden formar estructuras de triple-hélice estables y superestructuras fibrosas complejas que son responsables de las características retráctiles y de la densidad de los tejidos [Canty y Kadler 2005, Heino et al. 2009, Herr y Farndale 2009]. Los COLs fibrilares tipos I y II (COL1 y COL2) constituyen la forma mayoritaria de colágeno [Wess 2005], siendo el COL1 predominante en tejidos/órganos no-cartilaginosos tales como p.e. tendones, hueso, piel, córnea, tejido conectivo o las paredes de los vasos sanguíneos, donde normalmente se presenta como fibras heterotípicas [Wenstrup et al. 2004, Canty y Kadler 2005]. Fibroblastos, monocitos y células de otros tipos, como las endoteliales y epiteliales, son capaces de secretar de

forma eficiente proCOL *in vivo* [Lindblad 1998], el cual es procesado y ensamblado en la superficie celular [Canty y Kadler 2005].

El COL de la ECM interactúa con las células mediante diversos tipos de receptores específicos: ITGs [Zaman 2007], glicoproteína VI [Vogel 2001], receptores dominio discoidina (DDRs) [Shrivastava et al. 1997, Vogel et al. 1997], receptores tipo inmunoglobulina asociados a leucocitos (LAIRs) [Lebbink et al. 2006] y receptores de la familia de la manosa [Leitinger y Hohenester 2007]. Estos receptores al unirse con el COL pueden a su vez inducir la activación de otras moléculas (matrikinas, MMPs, TIMPs citoquinas y factores de crecimiento) indispensables para los procesos de remodelación, inflamación, repuesta inmune y reparación de tejidos [Garnotel et al. 2000, Heino 2000, Vogel 2001, Tam et al. 2004, Tran et al. 2004, Lebbink et al. 2006, Lee et al. 2007, Leitinger y Hohenester 2007, Farndale et al. 2008, Lebbink et al. 2008, Heino et al. 2009, Herr y Farndale 2009, Schultz y Wysocki 2009].

1.1.2 Integrinas

Las ITGs han sido ampliamente descritas como los principales receptores de superficie en células eucariotas [Hynes 2002, Delon y Brown 2007]. Actúan como vínculos transmembrana entre los contactos extracelulares (otras células o la ECM) y los microfilamentos de actina del citoesqueleto, los cuáles a su vez regulan y modulan la actividad de las ITGs [Takada et al. 2007]. En vertebrados, las subunidades de las ITGs se han diversificado desde secuencias y dominios preexistentes en procariotas y metazoos primitivos, en estrecha relación con los grandes cambios ocurridos durante la evolución en los cordados, tales como la transición de las estructuras cartilaginosas hacia las óseas, la aparición de un sistema inmunitario dual (innato y adaptativo) y el desarrollo de un sistema circulatorio de alta-presión, que caracterizan al linaje de los vertebrados [Johnson et al. 2009].

Estos receptores se agrupan en una superfamilia de αβ heterodímeros implicados en numerosos procesos celulares (migración, adhesión, diferenciación,

proliferación, fibrosis, invasión, tráfico leucocitario, hemostasis y respuesta inmunitaria) y en la reparación y remodelación de tejidos, así como también en enfermedades autoinmunes y en cáncer [Sueoka et al. 1997, Datta et al. 2002, Hynes 2002, Larsen et al. 2006, Delon y Brown 2007, Lathia et al. 2009].

Cuatro de los heterodímeros de ITGs beta 1 (α1β1, α2β1, α10β1 y α11β1) actúan principalmente como receptores de COL a través del dominio-I (también llamado A por su homología con el dominio Von Willebrand factor A) que es capaz de unirse directamente al COL y sólo se encuentra presente en vertebrados, posiblemente debido a una co-evolución receptor-ligando [Huhtala et al. 2005, Leitinger y Hohenester 2007, Takada et al. 2007, Johnson et al. 2009].

La unión ITG-COL desempeña un importante papel modulador a lo largo del desarrollo embrionario y la organogénesis [Hynes 2002, Johnson et al. 2009] así como en la transmisión de señales durante la generación de cartílagos y los procesos de reparación tisular, especialmente por inducción de la producción de MMPs e incremento en la adhesión celular [Pilcher et al. 1999, Ivkovic et al. 2003, Ronzière et al. 2005, Grenache et al. 2006, Mócsai et al. 2006, Zhang et al. 2006, Djouad et al. 2007, Zweers et al. 2007, Krane e Inada 2008].

1.1.3 Metaloproteasas de la matriz

La proteólisis extracelular es un paso esencial, tanto en procesos normales o fisiológicos como patológicos, en el que intervienen varias familias de enzimas, especialmente las endopeptidasas dependientes de zinc conocidas como MMPs [Ikejiri et al. 2005, Verma y Hansch 2007]. Desde un principio las MMPs se han descrito como factores colagenolíticos que pueden ser liberados o acumulados por las células en función de sus necesidades. Estas enzimas son reguladas por inhibidores endógenos o por la modulación de la síntesis *de novo* [Gross y Lapiere 1962, Woessner 1991, Butler y Overall 2009]. El estudio de estas enzimas ha despertado un gran interés por su participación en diversas enfermedades en humanos, especialmente en cáncer,

aunque su papel en condiciones normales no ha sido completamente determinado [Stefanidakis y Koivunen 2006, Page-McCaw et al. 2007], en parte por la falta de conocimientos sobre los sustratos y las rutas controladas por la acción de las MMPs [Butler y Overall 2009].

Las MMPs secretadas en el espacio extracelular son capaces de degradar proteínas de la ECM y de otras moléculas [Woessner 1991, Butler y Overall 2009] entre las que se incluyen factores de crecimiento, moléculas de adhesión celular, citoquinas, quimioquinas, otras MMPs y proteasas, inhibidores de proteasas, receptores celulares de superficie y factores aglutinantes sanguíneos [Stefanidakis y Koivunen 2006, Page-McCaw et al. 2007, Verma y Hansch 2007]. Incluso, la unión de MMPs con proteínas de superficie puede afectar la señalización intracelular, promover la internalización de enzimas o mediar la motilidad de células por disrupción de los enlaces con moléculas de la ECM [Stefanidakis y Koivunen 2006, Page-McCaw et al. 2007, Butler y Overall 2009].

Actualmente se han identificado 29 MMPs, de las cuáles MMP-2, -9 y -13 son de las más estudiadas en vertebrados por su participación en diferentes procesos y rutas de señalización. La MMP-2 participa en los mecanismos de maduración de proCOL, angiogénesis y respuesta inmunitaria [Amălinei et al. 2007, Butler y Overall 2009]. La MMP-9 está más relacionada con la modulación de la adhesión celular [Butler y Overall 2009, Stevens et al. 2009]. La MMP-13 es capaz de degradar COLs fibrilares (tipo I, II y III), sobre los que entonces pueden actuar las MMP-2 y -9; procesamiento clave para el desarrollo condral, osificación y reparación tisular [Inada et al. 2004, Stickens et al. 2004, Amălinei et al. 2007, Blumer et al. 2008, Takaishi et al. 2008]. Asimismo, estas MMPs tienen una acción sinérgica en los procesos inflamatorios, reclutamiento vascular, modulación de la ECM, remodelación ósea y quimiotaxis [Inada et al. 2004, Stickens et al. 2004, Amălinei et al. 2007, Takaishi et al. 2008, Butler y Overall 2009, Stevens et al. 2009].

1.1.4 Inhibidores Tisulares de MMPs

El balance entre los niveles de expresión y actividad de MMPs/TIMPs es un aspecto crítico para el normal desarrollo en los vertebrados [Rolland et al. 1998, Zhang et al. 2003, Crawford y Pilgrim 2005, Smith et al. 2006, Bednarek et al. 2009, Wyatt et al. 2009] siendo determinante en algunos mecanismos tales como la activación de moléculas del sistema inmunitario [Werb 1997, Mott y Werb 2004, Stevens et al. 2009] o la adecuada remodelación de la ECM a través del procesamiento de sus componentes estructurales [Mott y Werb 2004, Blumer et al. 2008, Stevens et al. 2009]. La pérdida de este balance se ha relacionado con condiciones clínicas como p.e. cáncer, sepsis, artritis, úlceras tisulares y fibrosis [Sternlicht y Werb 2001, Ljumovic et al. 2004, Chirco et al. 2006, Smith et al. 2006, Amălinei et al. 2007, Huxley-Jones et al. 2007, Yu et al. 2008, Del Casar et al. 2009, Lorente et al. 2009, Rosenberg 2009].

La alta actividad gelatinolítica en tumores ha hecho que el estudio de los TIMPs como inhibidores endógenos de gelatinasas sea de particular interés en la búsqueda de interacciones específicas [Ikejiri et al. 2005, Stefanidakis y Koivunen 2006, Verma y Hansch 2007, Del Casar et al. 2009]. En mamíferos se ha descrito una afinidad diferenciada entre MMPs y TIMPs, p.e. TIMP-1 es más eficiente en la inhibición de MMP-1, -3 y -9, mientras que TIMP-2 lo es para MMP-2 [Woessner 1991, Smith et al. 2006] y MMP-1 de membrana (MT-MMP1) [Sternlicht y Werb 2001].

Por otra parte, la filogenia de los TIMPs revela que esta familia ha evolucionado en vertebrados desde un ancestro común a través de duplicaciones de genes pre-existentes [Yu et al. 2003, Huxley-Jones et al. 2007]. En el urocordado *Ciona intestinalis* se ha identificado solo un TIMP ortólogo a los cuatro TIMPs descritos en humanos (TIMP-1, -2, -3 y -4), mientras que en el pez teleósteo *Danio rerio* el gen ortólogo al TIMP-1 está ausente pero hay representantes de los otros tres TIMPs, incluyendo duplicados de los TIMP-2 y TIMP-4 [Wyatt et al. 2009].

1.2 EL SISTEMA INMUNITARIO

La respuesta inmunitaria constituye un mecanismo de defensa frente a agentes (p.e. virus, bacterias, hongos, protozoos y parásitos multicelulares) o sustancias (p.e. ácidos nucleicos, proteínas y polisacáridos) que resultan ajenas al organismo, sin implicar la consecuencia patológica de tal reacción [Abbas et al. 2001]. El sistema inmunitario está formado por células y moléculas coordinadas capaces de generar una gran variedad de respuestas inmunitarias acorde con la diversidad de agentes infecciosos [Male y Roitt, 1996]; y en general, la respuesta inmunitaria se puede dividir en dos ramas: innata (natural o inespecífica) y adaptativa (adquirida o específica).

La respuesta innata incluye barreras físicas, células fagocíticas y células citotóxicas naturales, varias moléculas de la sangre (complemento y proteínas de la fase aguda), etc. [Male y Roitt 1996, Abbas et al. 2001], que actúan como una primera defensa ante infecciones o daños [Mollen et al. 2006]. La respuesta adaptativa incluye a linfocitos y los anticuerpos que secretan, y aparece exclusivamente en los vertebrados [Abbas et al. 2001]. Los linfocitos son capaces de reconocer patógenos individuales, para lo que existen dos categorías principales de células: los linfocitos T (células T) y los linfocitos B (células B). Las células B se encargan de reconocer y combatir patógenos extracelulares y sus productos, mediante anticuerpos que se unen específicamente a una molécula diana o antígeno; mientras las células T realizan actividades complementarias como la regulación de la producción de anticuerpos o la destrucción de patógenos fagocitados.

Asimismo, en la regulación de la respuesta inmunitaria participan las citoquinas [Vilcek 1997], moléculas que median gran parte de las fases efectoras de la inmunidad innata y la adaptativa [Abbas et al. 2001]. Las citoquinas ejercen múltiples funciones ente las que se incluyen la regulación de la proliferación y diferenciación de poblaciones linfocitarias, así como también la activación y regulación de las células inflamatorias (fagocitos mononucleares, neutrófilos y eosinófilos). La secreción de citoquinas es breve y autolimitada, y su síntesis se inicia por una nueva transcripción

génica. En general, las citoquinas inician su acción uniéndose a receptores de superficie presentes en la célula diana, los cuales tienen una elevada afinidad y su expresión es regulada por señales específicas (otra citoquina o incluso la misma citoquina). [Roca 2009]

1.2.1 El sistema inmunitario en peces teleósteos

En peces teleósteos el sistema inmunitario muestra características similares al de aves y mamíferos, con respuestas celulares y humorales que poseen las características de especificidad y memoria [Van Muiswinkel 1995]. Su respuesta innata consta de barreras físicas (epitelios y mucosas), efectores celulares (células fagocíticas y citotóxicas no específicas) y factores humorales (complemento y otras proteínas de la fase aguda). Mientras su respuesta adaptativa engloba un componente celular (linfocitos) y otro humoral (anticuerpos). Sin embargo, pese a sus semejanzas con el sistema inmunitario de otros vertebrados, existen diferencias claras como p.e. que los peces dependen en mayor grado de los mecanismos de defensa innatos que los mamíferos, principalmente en condiciones ambientales de baja temperatura (los peces son animales poiquilotermos) puesto que la respuesta inmunitaria adaptativa es dependiente de la temperatura [Cuchens y Clem 1977, Avtalion 1981, Abruzzini et al. 1982, Clem et al. 1984, 1991, Miller y Clem 1984].

Los órganos y tejidos del sistema inmunitario en teleósteos se han clasificado, como en mamíferos, en órganos primarios y secundarios [Zapata et al. 1996]. Entre los órganos primarios, el riñón constituye su órgano hematopoyético por excelencia y consta de dos partes, (1) la anterior o cefálica, con función fundamentalmente hematopoyética y, (2) la posterior, con función básicamente excretora. Mientras que el bazo es el órgano linfoide secundario más importante, aunque presenta pocos linfocitos que pueden incrementarse mediante la administración de un antígeno [Roca 2009].

Después de las escamas, la piel y el mucus que actúan como la primera barrera defensiva, la respuesta celular innata de los peces incluye una gran variedad de leucocitos. Entre ellos se incluyen los fagocitos (monocitos/MCs y granulocitos) y las células citotóxicas no específicas [Secombes 1996]. Los fagocitos serían de mayor importancia para la inmunidad innata ya que se encargan de eliminar virus, bacterias y parásitos [Rowley et al. 1988, Secombes y Fletcher 1992, Sepulcre et al. 2002], además de que podrían ser los iniciadores de la activación y regulación de la respuesta inmunitaria específica [Clem et al. 1985, 1991, Vallejo et al. 1992].

En dorada (*Sparus aurata* L.) se ha descrito que los granulocitos acidófilos (AGs) son las células fagocíticas más activas y abundantes, con funciones similares a las de los neutrófilos de humanos [Sepulcre et al. 2002]. El proceso de fagocitosis por leucocitos de peces presenta las mismas etapas descritas en mamíferos, finalizando con dos mecanismos responsables de la muerte de los microorganismos fagocitados, 1) la producción de intermediarios reactivos de oxígeno (ROIs) y, 2) la producción de óxido nítrico (NO) y otros intermediarios reactivos de nitrógeno (RNIs). Además, se conoce que los ROIs producidos durante la explosión respiratoria en estos fagocitos tienen actividad bactericida [Sharp y Secombes 1993, Skarmeta et al. 1995].

El sistema inmunitario adaptativo de teleósteos, al igual que el de mamíferos, presenta memoria [Van Muiswinkel 1995]. Tras un primer contacto con el antígeno se produce un menor título de anticuerpos específicos en el suero, que el producido en posteriores contactos (respuesta secundaria), siendo esta respuesta dependiente de la temperatura. La iniciación de la respuesta inmunitaria adaptativa está controlada por los MCs que funcionan como células presentadoras de antígenos para los linfocitos [Clem et al. 1985, Vallejo et al. 1992]. Los linfocitos a su vez producen citoquinas que activan a los MCs tras ser estimulados con un antígeno [Graham y Secombes 1988]. De esta manera se mantiene una coordinación y regulación mutuas entre la respuesta inmunitaria innata y la adaptativa.

1.2.2 Regulación de la respuesta inmunitaria por moléculas de la ECM

Las citoquinas y los factores de crecimiento pueden estimular la diferenciación celular inducida por la ECM y coordinada por dominios bioactivos de glicoproteínas de la matriz como p.e. COL, laminina y fibronectina [Schnaper y Kleinman 1993, Vernon y Gooden 2002, Ågren y Werthén 2007, Leitinger y Hohenester 2007]. El COL ha sido reconocido como un potencial regulador de la respuesta inflamatoria en mamíferos [Pacifici et al. 1991] siendo sus actividades biológicas reguladas mediante varios receptores específicos, como las ITGs α1β1 y α2β1 [Zaman 2007]. En mamíferos, algunos de estos receptores se expresan en MCs y células dendríticas, y al unirse al COL pueden resultar en la activación [Pacifici et al. 1991, Matsuyama et al. 2004, Lee et al. 2007] o inhibición [Lebbink et al. 2006] de estos tipos celulares. Así, se ha descrito que un estricto control sobre la señalización de ITGs es necesario para una coordinación apropiada del sistema inmunitario innato y los procesos inflamatorios [Mócsai et al. 2006].

Asimismo, las MMPs también son capaces de activar o degradar una gran variedad de sustratos no relacionados con la ECM tales como quimioquinas, citoquinas, factores de crecimiento y proteínas de unión. Por ello se las ha reconocido como un elemento clave en la respuesta inflamatoria, participando en la regulación de las funciones de defensa innata y adquirida del hospedero [Ravi et al. 2007, Lorente et al. 2009]. En este contexto, es de gran importancia el estricto control de la actividad enzimática de las MMPs por sus inhibidores endógenos, los TIMPs [Ravi et al. 2007, Verma y Hansch 2007]. La pérdida de balance MMPs/TIMPs se traduce en diversos desórdenes patológicos incluyendo alteraciones en los procesos de reparación tisular, angiogénesis, apoptosis, etc. [Verma y Hansch 2007], por lo cual se ha sugerido su utilización como biomarcadores en cuadros infecciosos [Lorente et al. 2009].

De esta manera, la ECM es un componente activo en la respuesta inmunitaria y actúa, además, como un reservorio especializado de factores que promueven la proliferación, activación y migración celular. Esto influiría en el desarrollo de la respuesta inflamatoria [Vaday y Lider 2000, Morwood y Nicholson 2006], al

proporcionar las señales necesarias para dirigir y coordinar la migración - adhesión de los leucocitos hacia sus lugares de destino [Vaday et al. 2001].

En peces, la respuesta inmunitaria innata ha sido relativamente bien caracterizada a nivel celular y molecular en los últimos años, sin embargo existen pocos trabajos sobre el papel de la ECM en la regulación de la inmunidad innata e inflamación que requeriría un mayor análisis y estudio.

1.3 EL SISTEMA REPRODUCTOR

El sistema reproductor en los vertebrados es muy variable atendiendo a la gran diversidad de estrategias de reproducción que incluyen importantes procesos de remodelación tisular. En este sentido, el entorno de la ECM y las moléculas que lo componen tienen un papel relevante, no solo desde el punto vista estructural, sino que también intervienen activamente en la señalización de los mecanismos específicos que regulan el sistema reproductor.

1.3.1 El sistema reproductor en peces teleósteos

El sistema reproductor en peces resulta complejo de generalizar si se lo compara con otros vertebrados, debido a que este grupo presenta una gran diversidad de mecanismos de reproducción, incluyendo los procesos de fecundación y la periodicidad con estos ocurren, mismos que a su vez dependen de otros factores como p.e. las condiciones ambientales (fotoperíodo, temperatura, osmolaridad, disponibilidad de alimento, etc.). En este sentido, existen especies que desarrollan un único sexo funcional a lo largo de su vida (gonocoristas) y especies que presentan ambos sexos funcionales a lo largo de su vida (hermafroditas).

Las especies hermafroditas pueden ser protogínicas o protándricas en función del sexo que desarrollen antes, o pueden ser ambisexuales. Por ejemplo, en el pez

protógino *Synbranchus marmoratus* los individuos machos pueden desarrollarse inicialmente como tales o ser el resultado de la posterior reversión sexual de las hembras, implicando procesos de remodelación tisular y modificaciones en las poblaciones celulares [Lo Nostro et al. 2004]. Sin embargo, la dorada es una especie hermafrodita protándrica en la cual los individuos son machos funcionales durante los dos primeros ciclos reproductivos (RCs), pudiendo posteriormente pasar a hembras [Chaves-Pozo et al. 2005a]. A pesar de esta gran diversidad reproductiva, las características morfológicas y funcionales de la gónada de teleósteos están muy conservadas, incluso en las especies hermafroditas [Le Gac y Loir, 1999].

Los ciclos reproductivos de los teleósteos se pueden clasificar en función de la longitud del periodo de puesta. Así, hay especies como (1) los salmónidos, características de clima frío, que ponen durante varias semanas al año, especies como (2) la dorada y la carpa común, características de clima templado, que ponen durante varios meses al año y (3) especies características de clima tropical, que ponen durante todo el año. Este comportamiento se ve reflejado en el ciclo gonadal ya que en las especies de puesta estacional este ciclo se puede dividir en cuatro fases: gametogénesis, puesta, post-puesta y quiescencia, mientras que las especies que ponen durante todo el año carecen de las fases de post-puesta y quiescencia (Scott 1987, Miura 1999).

La dorada, siendo una especie hermafrodita protándrica de puesta estacional, presenta durante el primer RC las cuatro fases mencionadas, sin embargo en el segundo RC la etapa de quiescencia es sustituida por una involución testicular que les puede llevar a su conversión en hembras. Durante estas etapas, especialmente en las etapas de post-puesta e involución testicular, la gónada sufre importantes cambios morfológicos fruto de una importante remodelación tisular [Chaves-Pozo et al. 2005a, Liarte et al. 2007).

1.3.2 Regulación de la espermatogénesis por medio de moléculas de la ECM

En epitelios endocrino-dependientes, como los túbulos seminíferos de roedores, se ha descrito que la membrana basal (una forma especializada de la ECM) interactúa con factores de crecimiento y hormonas para mantener las funciones celulares específicas de los túbulos [Dym 1994]. Se sabe que la ITGB1 (uno de los principales receptores de COL) desempeña un papel relevante en la especialización ectoplasmática en mamíferos, participando en las uniones adherentes y estrechas entre las células germinales y células de Sertoli durante la espermatogénesis [Siu et al. 2003]. A su vez se ha demostrado que en células de Sertoli estimuladas con componentes purificados de la ECM se produce un incremento de la secreción de la proteína de unión a andrógenos y la transferrina, efecto que se potencia en presencia de suero [Dym 1994].

El papel de la ECM en el sistema reproductor también se pone de manifiesto por su particular composición, p.e. en mamíferos la MMP-23 se expresa principalmente en tejidos reproductivos [Amălinei et al. 2007] y el COL26 solo se expresa en testículo y ovario [Myllyharju y Kivirikko 2004]. En el pez protógino *Synbranchus marmoratus*, las fibras de COL en el testículo difieren en abundancia y orientación entre los grupos de machos primarios y los resultantes del proceso de reversión sexual (secundarios) [Lo Nostro et al. 2004].

En el ovario de mamíferos las células de la granulosa (homólogas con las células de Sertoli del testículo) incrementan la producción de proteínas de la zona pelúcida cuando son cultivadas en un sustrato de ECM [Dym 1994]. En *Drosophila* la interacción entre COL4 y un homólogo de la proteína morfogénica ósea (BMP) es crucial en la regulación del número de células madre germinales en ovario [Hynes 2009]. También las MMPs están implicadas en la fisiología ovárica (siendo su expresión regulada por hormonas, factores de crecimiento y citoquinas) [Verma y Hansch 2007] y participan en el control de la ovulación, implantación embrionaria, involución uterina, mamaria y prostática, menstruación y proliferación endometrial [Amălinei et al. 2007].

Adicionalmente se ha descubierto que esteroides sexuales como el 17β-estradiol (E2) son cruciales como moduladores en casos de desregulación de la remodelación de la ECM tanto *in vivo* como *in vitro* [Elliot et al. 2009]. La progesterona incrementa los niveles de expresión de las subunidades de ITGs α4, α5 y β1 pero no las de la ITG αVβ3 implicada en la implantación y que a su vez es inhibida en presencia de estrógeno [Bowen y Hunt 2000].

En general, la amplia gama de interacciones a nivel celular y molecular con los componentes de la ECM sugieren una profunda implicación del entorno de la ECM durante la señalización de las diferentes rutas y la comunicación entre las diferentes moléculas y sus receptores. Los dominios ordenados en las moléculas de la ECM promoverían la formación de complejos de receptores organizados en la superficie de las membranas, estos complejos actuarían como reguladores en la membrana-proximal y promoverían la integración de las señales traducidas, en un paralelismo con los complejos de receptores inmunoreguladores durante la sinapsis inmunológica (en la que también existen interacciones entre integrinas y otros receptores) [Hynes 2009].

En el presente trabajo hemos utilizado la dorada como animal de experimentación debido a sus características biológicas de reproducción estacional, involución testicular y reversión sexual, muy útiles en el estudio de las interacciones entre los sistemas inmunitario y reproductor. De estudios previos en nuestro grupo de investigación conocemos que en riñón cefálico los AGs [Sepulcre et al. 2002] y los MCs son las principales células fagocíticas, pudiéndose regular sus actividades mediante diferentes patrones moleculares asociados a patógenos (PAMPs) como el lipopolisacárido de enterobacterias (LPS) o el ADN genómico de *Vibrio anguillarum* (VaDNA) [Sepulcre et al. 2007]. En la gónada se produce una infiltración de AGs en las etapas de post-puesta e involución testicular, donde parecen participar más en la regulación del ciclo celular testicular que en la eliminación de células degenerativas [Chaves-Pozo et al. 2005a].

Asimismo, se ha propuesto que los AGs infiltrados participarían en la en la morfogénesis testicular de juveniles y machos, colaborando en la producción de interleuquina 1 beta (IL-1β) como un factor proliferativo en células germinales de testículo [Chaves-Pozo et al. 2009]. En este sentido, los AGs se han descrito como fagocitos profesionales capaces de adaptarse a un microambiente y desarrollarse en subgrupos funcionales [Chaves-Pozo et al. 2005b]. También se ha demostrado que los AGs de testículo presentan actividad gelatinolítica correspondiente a MMP-2 y MMP-9, mientras que en los AGs de riñón cefálico solo se detecta la actividad correspondiente a MMP-2. Estos datos junto al perfil de expresión de MMP-9, MMP-13, TIMP-2a y TIMP-2b en AGs de testículo y su regulación por 17β-estradiol (E2) sugieren un papel relevante de las MMPs en la fisiología gonadal y la infiltración de AGs durante la post-puesta [Chaves-Pozo et al. 2008].

Teniendo en cuenta los datos previos, en este trabajo se ha realizado un estudio sobre el papel de las moléculas relacionadas con la ECM en la respuesta inmunitaria y en la espermatogénesis de la dorada.

2 OBJETIVOS

2.1. Determinar si el COL es capaz de modular la actividad de las células fagocíticas profesionales de la dorada.

2.2. Estudiar las correlaciones existentes entre el perfil de expresión de MMPs, TIMPs, COLs e ITGs como moléculas relacionadas con la ECM y caracterizar su modulación en la respuesta inflamatoria de la dorada.

2.3. Estudiar la modulación de la actividad de células SAF-1 (fibroblastos de aleta de dorada) mediada por secuencias específicas derivadas de COL, durante los procesos iniciales de reparación tisular e inflamación.

2.4. Caracterizar el perfil de expresión génica de la ITGB1b (gen ortólogo al de la ITGB1 de mamíferos) y su modulación durante el ciclo reproductivo de la dorada.

3 PRINCIPALES RESULTADOS Y DISCUSIÓN

3.1 El colágeno regula la activación de fagocitos profesionales en el pez teleósteo dorada

Aunque en peces la respuesta inmune innata contra infecciones se encuentra relativamente bien caracterizada a nivel celular y molecular, su regulación a través de moléculas relacionadas a la ECM ha sido muy poco estudiada hasta ahora. Nosotros en este trabajo [Castillo-Briceño et al. 2009] hemos evaluado el efecto del COL en sus formas nativa y desnaturalizada (gelatina) en la explosión respiratoria de fagocitos de riñón cefálico, encontrándose que ambas formas incrementan la producción de ROIs de forma dosis dependiente y comparable a la estimulación con PAMPs (LPS/VaDNA). Estos resultados nos llevaron a analizar los perfiles de expresión génica de moléculas relevantes en la respuesta inmunitaria o proteínas relacionadas con la ECM en AGs y MCs estimulados con COL1 (10 μg/ml) y gelatina (10 μg/ml), en comparación con los estimulados con LPS (10 μg/ml) / VaDNA (50 μg/ml), resultando que ambas formas de COL inducen un grupo específico de genes relacionados con las respuesta inmunitaria y enzimas de la ECM. Este perfil de expresión es sustancialmente diferente del obtenido con los PAMPs, así COL y gelatina inducen la expresión de IL-1β, la quimioquina CCL4, MMP-9 y MMP-13 en AGs y MCs, pero no modulan de forma significativa otros genes pro-inflamatorios que son drásticamente incrementados con los PAMPs como ciclooxigenasa 2 e IL-6. Asimismo, se encontró que el inhibidor V de MMP-2/MMP-9 inhibe de forma dosis dependiente la activación de fagocitos con ambas formas de COL, mientras que si son pretratadas con colagenasa se incrementa su capacidad estimulante.

Todos estos resultados indicarían que la activación de fagocitos mediada por el COL es incrementada cuando éste se encuentra fragmentado, ya sea por la acción de diferentes proteasas o por factores físicos, siendo los fagocitos de peces capaces de reconocer estos fragmentos. En consecuencia, se propone que en peces además de la

bien establecida respuesta contra infecciones, el sistema inmunitario innato sería también capaz de responder de forma diferenciada ante señales de daño tisular (DAMPs).

3.2 Correlaciones en el perfil de expresión de moléculas relacionadas con la matriz extracelular durante la respuesta inflamatoria en dorada

Además de sus funciones estructurales, los componentes de la ECM interactúan con receptores de la superficie celular y componentes intracelulares para modular la transmisión de señales de crecimiento celular, diferenciación, migración, proliferación, polarización, apoptosis e inflamación. En nuestros resultados previos en dorada demostramos que (1) tanto estímulos de tipo endocrino como los relacionados con la respuesta inmunitaria modulan la expresión de MMPs y TIMPs así como la actividad gelatinolítica en testículo [Chaves-Pozo et al. 2008a], y (2) que el COL1 en suspensión induce la expresión de un subgrupo específico de citoquinas pro-inflamatorias y MMPs en fagocitos profesionales [Castillo-Briceño et al. 2009], sugiriendo una modulación coordinada de las moléculas relacionadas con la ECM durante la respuesta inflamatoria. En consecuencia, en este trabajo [Castillo-Briceño et al. 2010] hemos analizado los perfiles de expresión de genes que codifican diversos tipos de moléculas relacionadas con la ECM como MMP-2, MMP-9, MMP-13, TIMP-2a, TIMP-2b, COL1A1 y la ITGB1a en diferentes órganos de animales adultos, así como su modulación en respuesta a pruebas de desafío de la respuesta inmunitaria. Los resultados mostraron que en hígado la expresión de estos genes se encuentra siempre en el grupo de órganos con menores niveles de expresión, sin embargo la expresión de todos estos genes se incrementó significativamente ante una lesión inducida, resultando un perfil diferente al obtenido tras inducir una infección. Asimismo se sugiere que las moléculas relacionadas con la ECM participarían activamente en los procesos pro-inflamatorios, con un papel particular para el COL1 que sería sintetizado por células inmunitarias activadas y sobre las que sería capaz de actuar como un estímulo autocrino/paracrino. Finalmente, estos resultados sugieren que las correlaciones entre moléculas relacionadas a la ECM podrían servir como biomarcadores en procesos iniciales de

inflamación, de forma similar a los propuesto para las correlaciones entre MMPs y otras moléculas del entorno de la ECM como un medio de evaluación efectivo en casos de endometriosis [Salata et al. 2008] y daño del miocardio [Ceauşu et al. 2009] en humanos. En este sentido, el estudio de las correlaciones entre las moléculas relacionadas con la ECM y otros parámetros relacionados con las enfermedades sería un parámetro útil para la caracterización/detección de cuadros patofisiológicos no detectados mediante la medición del incremento o proporciones relativas comúnmente utilizadas.

3.3 Implicación de secuencias específicas de colágeno durante los procesos de inflamación y reparación tisular en fibroblastos de dorada

Los sitios y secuencias específicas de COL a los cuáles las células se adhieren, ya sea de forma directa o a través de proteínas intermediarias, han sido identificados utilizando baterías de péptidos derivados de COL con estructura de triple hélice que mimetizan las fibras de COL y contienen secuencias o motivos específicos GXX'GEX'' [Knight et al. 2000, Raynal et al. 2006, Farndale et al. 2008, Boilard et al. 2010], donde X, X' y X'' representan residuos aminoacídicos intercambiables. Los niveles de afinidad diferentes entre motivos específicos los hacen capaces de controlar la adhesión y migración de las células independientemente de la regulación intracelular. Previamente demostramos que en dorada el COL1 es capaz de disparar la respuesta respiratoria *in vitro* e inducir un grupo específico de genes relacionados con la respuesta inmune y del entorno de la ECM en fagocitos [Castillo-Briceño et al. 2009], pudiendo actuar como una señal intermedia durante la respuesta inflamatoria inicial ante señales de daño e infección [Castillo-Briceño et al. 2010]. En este sentido, ya que los fibroblastos están altamente relacionados en los procesos iniciales de reparación y regeneración de tejidos, utilizamos la línea celular SAF-1 (fibroblastos de aleta caudal de dorada) para identificar motivos de COL de alta afinidad para la adhesión celular en peces teleósteos. Para ello se realizaron ensayos de adhesión a tiempo final y a tiempo real con baterías de péptidos de COL y motivos específicos, identificándose aquellos implicados en la adhesión inicial de los fibroblastos de manera magnesio-dependiente,

siendo esta adhesión inhibida en presencia de EDTA e inespecíficamente incrementada en presencia de VaDNA. Concretamente, se encontró que los fibroblastos de dorada presentan una muy alta afinidad de adhesión con los motivos GFOGER y GLOGEN usados como sustrato (donde O es hidroxiprolina), tanto en cantidad de células como en extensión de las mismas, mientras que en suspensión son capaces de inducir la expresión de genes codificantes de moléculas pro-inflamatorias como IL-1β y ciclooxigenasa 2. Asimismo, el análisis bioinformático de estos motivos en complejo con la el dominio tipo I de la ITGB1a muestran una dinámica similar a la encontrada con el dominio I de las ITGA típicamente consideradas como el principal receptor de unión celular a estos motivos en mamíferos de una forma magnesio-dependiente. Estos datos en conjunto indicarían una implicación relevante de las secuencias específicas de COL en la regulación de la respuesta inicial inflamatoria y de reparación tisular en peces teleósteos, que estaría mediada por la unión COL-ITG, modificando la mecánica celular y la señalización posterior.

3.4 Una isoforma de la ITGB1 exclusivamente expresada en testículo, cerebro y células endoteliales, es modulada durante el ciclo reproductivo en la dorada

El descubrimiento de receptores celulares de superficie que son capaces de interactuar con los componentes del entorno de la ECM, ha dado paso a un creciente interés por el estudio de las moléculas en el compartimento extracelular. Este compartimento presentaría una composición y organización cambiante a través del desarrollo, interactuando con diversos componentes intracelulares que modulan la transmisión de señales de crecimiento, diferenciación, migración, apoptosis, etc. Los hallazgos previos en dorada mostraron una modulación de las moléculas relacionadas con la ECM en respuesta estímulos endocrinos e inmunitarios, con correlaciones específicas durante la respuesta inflamatoria inicial. En este trabajo, hemos efectuado una caracterización inicial de la expresión génica mediante RT-qPCR de la ITGB1b, una isoforma que parece estar implicada en los procesos de espermatogénesis en testículo. Para comprobarlo se analizó su perfil de expresión se analizó en relación con los perfiles de otras moléculas relacionadas a la ECM tales como MMP-2, MMP-9, MMP-

13, TIMP-2a, TIMP-2b, COL1A1 e ITGB1a en las diferentes fases del RC. Los resultados mostraron que a diferencia de la ITGB1a que se expresa de forma constitutiva en todos los tejidos y desde el primer día después de la eclosión, la ITGB1b no fue detectada durante las fases larvaria y de juvenil, y su expresión en ejemplares adultos se restringe principalmente a testículo, cerebro y células endoteliales. El testículo presenta, en las fases de espermatogénesis y post-puesta del segundo RC, incrementos específicos de las moléculas relacionadas con la ECM, incluyendo la ITGB1a, la mayoría correlacionadas significativamente entre sí y con valores más elevados en la post-puesta, pero que difieren del perfil encontrado en el primer RC. Sin embargo, la ITGB1b presenta en ambos RCs un único incremento drástico que en ambos casos ocurre durante la espermatogénesis.

Estos resultados, constituirían un indicador inicial de una diferente funcionalidad de ambas formas de ITGB1 detectadas en dorada, donde la ITGB1a tendría una participación constitutiva comparable a la ITGB1 de mamíferos, mientras que la ITGB1b parecería estar más relacionada con los procesos endocrinos, especialmente con la espermatogénesis, como un complemento o especialización del papel de la ITGB1a en dorada.

Para complementar esta información y con el fin de analizar el posible efecto de la estimulación con hormonas sexuales sobre la expresión génica de la ITGB1b en testículo, estamos llevando a cabo experimentos *in vivo* que se encuentran en diferente grado de desarrollo, durante los cuales se han expuesto ejemplares adultos de dorada a niveles suprafisiológicos de testosterona (como estímulo androgénico) o EE2 (como disruptor de andrógenos), tomándose muestras a diferentes tiempos para su posterior procesamiento.

4 CONCLUSIONES

4.1. La dorada es capaz de responder de forma específica ante señales de daño a nivel celular y molecular, con un perfil diferente al de la respuesta establecida ante infecciones o PAMPs.

4.2. El COL nativo, los fragmentos y péptidos sintéticos derivados de COL presentes en suspensión son capaces de actuar como señales de daño (DAMPs), modulando la respuesta inmunitaria innata y los procesos de inflamación inicial en fagocitos profesionales y fibroblastos de dorada.

4.3. Los fibroblastos de dorada presentan una respuesta diferenciada durante la adhesión inicial para los diferentes motivos GXX'GEX" de COL, con mayor afinidad dependiente de magnesio para GFOGER y GLOGEN.

4.4. Las moléculas relacionadas a la ECM en dorada están altamente correlacionadas entre sí bajo condiciones fisiológicas normales y durante el desarrollo. Sin embargo, en AGs este grado de correlación es modulado en respuesta a DAMPs y PAMPs.

4.5. Los perfiles de expresión de moléculas relacionadas a la ECM en testículo de dorada muestran una mayor síntesis *de novo* durante la espermatogénesis, particularmente específica para la ITGB1b.

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CAPÍTULO II: IMPLICATION OF EXTRACELLULAR MATRIX RELATED MOLECULES IN THE IMMUNE RESPONSE AND SPERMATOGENESIS IN THE TELEOST FISH GILTHEAD SEABREAM

1. COLLAGEN REGULATES THE ACTIVATION OF PROFESSIONAL PHAGOCYTES OF THE TELEOST FISH GILTHEAD SEABREAM

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Collagen regulates the activation of professional phagocytes of the teleost fish gilthead seabream

Patricia Castillo-Briceño, María P. Sepulcre, Elena Chaves-Pozo, José Meseguer, Alfonsa García-Ayala*, Victoriano Mulero*

Department of Cell Biology and Histology, Faculty of Biology, University of Murcia, 30100 Murcia, Spain

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ABSTRACT

The innate immune system mediates the initial inflammatory response that follows infection or injury. Although the innate immune response of fish to infection has been relatively well characterized during recent years at both cellular and molecular levels, no studies have examined the role of extracellular matrix (ECM) in the regulation of innate immunity and inflammation. We report here that collagen and gelatin *in vitro* were able to prime the respiratory burst of phagocytes from the bony fish gilthead seabream. In addition, collagen and gelatin induced a specific set of immune-related and ECM remodelling enzymes that substantially differed from that induced by pathogen-associated molecular patterns. Notably, both collagen and gelatin induced the expression of interleukin-1β, chemokine (C–C motif) ligand 4 and matrix metalopeptidases (MMP) 9 and 13 in acidophilic granulocytes and macrophages but were unable to significantly increase the expression of other pro-inflammatory genes. Furthermore, it was found that the MMP2/MMP9 inhibitor V had a dose-dependent inhibitory effect on seabream phagocyte activation by either collagen or gelatin. In contrast, pre-treatment of collagen and gelatin by collagenase resulted in a higher stimulatory capacity compared to non-digested proteins. Collectively, these results indicate that collagen fragments produced by the action of different host proteases, and probably released by infectious agents, are sensed by fish phagocytes. Therefore, we propose that, besides to the well-established response to infection, the innate immune system of fish is able to respond to tissue injury.

1. Introduction

The innate immune system mediates the initial inflammatory response that follows infection or injury (Mollen et al., 2006). It has been proposed that both chemical and mechanical changes in the cellular microenvironment may be responsible for driving specific cellular responses (Jiang et al., 2007; Peyton et al., 2007). Thus, the extracellular matrix (ECM) not only provides structural support and adhesive substrates for the body tissues, but it also plays a significant role in regulating cell and tissue function (Schnaper and Kleinman, 1993).

ECM components and their mechanical properties induce changes in cell shape and movement, bind growth factors, and facilitate cell–cell and cell–ECM interactions. The ECM regulatory effects involve the modulation of signalling pathways that control cell growth, differentiation, proliferation, apoptosis, survival and morphogenesis (Lukashev and Werb, 1998; Peyton et al., 2007). The differentiation induced by ECM results from multiple stimuli such as: tensile forces on the cell, cytokine- or growth factor-mediated stimulation, and interaction with bioactive domains of matrix glycoproteins, such as collagen, laminin, fibronectin and hyaluronan (Schnaper and Kleinman, 1993; Vernon and Gooden, 2002; Ågren and Werthén, 2007; Leitinger and Hohenester, 2007).

The collagen fibrillar form is an abundant structural ECM protein that was very early recognized as a potential regulator of the inflammatory response in mammals (Pacifici et al., 1991). Collagen mediates its biological activities through interactions with different receptors, such as tyrosine kinases of the discoidin domain receptor (DDR) family (Shrivastava et al., 1997; Vogel et al., 1997), α1β1 and α2β1 integrins (Zaman, 2007), glycoprotein VI (Vogel, 2001), leukocyte-associated immunoglobulin-like receptor-1 (LAIR) (Lebbink et al., 2006), and members of the mannose receptor family (Leitinger and Hohenester, 2007). Notably, some of these receptors are expressed by mammalian macrophages and dendritic cells and their engagement by collagen may result in either the activation (Pacifici et al., 1991; Matsuyama et al., 2004; Lee et al., 2007) or the inhibition (Lebbink et al., 2006) of these cells.

Although the innate immune response of fish has been relatively well characterized during recent years at both cellular and molecular levels, no studies worked into the role of ECM in the regulation of innate immunity and inflammation. We have previously shown that gelatin is capable of activating acidophilic granulocytes from

* Corresponding authors. Tel.: +34 968 367581; fax: +34 968 363963.
E-mail addresses: agayala@um.es (A. García-Ayala), vmulero@um.es (V. Mulero).

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the gilthead seabream (*Sparus aurata* L.) (Chaves-Pozo et al., 2008). As these cells, together with macrophages, have been described as the professional phagocytes of this species, and their main activities have been found to be regulated by different pathogen-associated molecular patterns (PAMPs) (Sepulcre et al., 2007), we have studied the main functional activities and the gene expression profiles of these cells in response to collagen-stimulation and compared it with PAMP-stimulation.

2. Materials and methods

2.1. Fish

Healthy specimens (800 g mean weight) of gilthead seabream (*S. aurata* L.) (Actinopterygii, Perciformes, Sparidae), in their second reproductive cycle, were obtained from CULMAMUR, S.L. (Águilas, Spain). The fish were kept at the Spanish Oceanographic Institute (Mazarrón, Murcia) in 14 m^3 running seawater aquaria (dissolved oxygen 6 ppm, flow rate 20% aquarium volume/h) with natural temperature and photoperiod, and fed twice a day with a commercial pellet diet (Trouvit, Spain) at a feeding rate of 1.5% of fish biomass. Fish were fasted for 24 h before sampling. The experiments described comply with the Guidelines of the European Union Council (86/609/EU) and the Bioethical Committee of the University of Murcia (Spain) for the use of laboratory animals.

2.2. Cell isolation

Head kidneys were removed from fish specimens, dissociated through a 100 μm nylon mesh and adjusted to 10^7 viable cells/ml in sRPMI [RPMI-160 culture medium (Gibco) adjusted to gilthead seabream serum osmolarity (353.33 mosmol) with 0.35% NaCl] (Sepulcre et al., 2002) supplemented with 10% fetal calf serum (FCS, Gibco), 100 IU/ml penicillin and 100 μg/ml streptomycin (Biochrom).

Acidophilic granulocytes were obtained by magnetic-activated cell sorting (MACS) as described previously (Roca et al., 2006). Briefly, head kidney cell suspensions were incubated with a 1:10 dilution of a monoclonal antibody (mAb) specific to gilthead seabream acidophilic granulocytes (G7) (Sepulcre et al., 2002), washed twice with phosphate-buffered saline (PBS) containing 2 mM ethylenediaminetetraacetic acid (EDTA) (Sigma) and 5% FCS, and then incubated with 100–200 μl per 10^8 cells micro-magnetic-bead-conjugated anti-mouse IgG antibody (Miltenyi Biotec). After washing, $G7^+$ and $G7^-$ cell fractions were collected by MACS following the manufacturer's instructions and their purity was analysed by flow cytometry (Roca et al., 2006). Head kidney macrophage monolayers were then obtained after overnight culture of $G7^-$ fractions in FCS-free medium and their identity was confirmed by the expression of macrophage colony stimulating factor receptor (M-CSFR) (Roca et al., 2006).

2.3. Cell culture and treatments

Total head kidney leukocytes and macrophages and acidophilic granulocytes fractions were stimulated for 4 or 16 h at 23 °C with 10 μg/ml lipopolysaccharide (LPS, Sigma) and 50 μg/ml phenol-extracted genomic DNA from *Vibrio anguillarum* ATCC19264 cells (VaDNA), 0.01–10 μg/ml type I collagen from calf skin (C9791, Sigma) or 0.01–10 μg/ml gelatin from bovine skin (G9391, Sigma) in supplemented sRPMI. As type I collagen is acid-soluble and needs to be dissolved in 0.1 M acetic acid, a final concentration 0.001 M acetic acid was added to all samples. In some experiments, cells were pre-treated for 30 min with 20–200 nM MMP2/MMP9 inhibitor V (Cat. No. 444285, Calbiochem) or incubated for 16 h in the presence of 40 μg/ml polymyxin B (P4932, Sigma) before being stimulated as previously described. In another series of experiments, the culture plates were coated overnight at 4 °C with the stimuli, then treated for 10 min at 37 °C with 0.25–0.50 U/ml collagenase from *Clostridium histolyticum* (C5138, Sigma) in Hanks' balanced salt solution (HBSS, Gibco) and washed with PBS to remove the enzyme before adding the cells which were incubated for 16 h.

2.4. Reactive oxygen intermediate (ROI) production assays

Respiratory burst activity for head kidney cells was measured as the luminol-dependent chemiluminescence (Mulero et al., 2001) brought on by adding 100 μM luminol (Sigma) and 1 μg/ml phorbol myristate acetate (PMA) (Sigma). Then, the chemiluminescence was recorded every 117 s for 1 h in a FLUOstart luminometer (BGM, LabTechnologies). The values reported are the average of at least three fish, expressed as curve maximum, from which the apparatus background was subtracted.

2.5. Analysis of gene expression

Total RNA was extracted from cell pellets with TRIzol Reagent (Invitrogen) following the manufacturer's instructions and treated with DNase I, 1 U/μg RNA Amplification grade (Invitrogen). The SuperScript III RNase H^- ReverseTranscriptase (Invitrogen) was used to synthesize first strand cDNA with oligo-dT_{18} primer from 1 μg of total RNA at 50 °C for 50 min. Real-time PCR was performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures were incubated for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C, and finally 15 s at 95 °C, 1 min at 60 °C and 15 s at 95 °C. For each mRNA, gene expression was normalized to the ribosomal protein S18 content in each sample using the comparative Ct method ($2^{-\Delta\Delta Ct}$). The primers used are shown in Table 1. In all cases, each PCR was performed with triplicate samples and repeated with at least two independent samples.

Table 1
Primer sequences used for gene expression analysis. The gene symbols followed the Zebrafish Nomenclature Guidelines (http://zfin.org/zf_info/nomen.html).

Gene	Accession number	Primer name	Nucleotide sequence (5′ → 3′)
rps18	AM490061	F	AGGGTGTTGGCAGACGTTAC
		R	CTTCTGCCTGTTGAGGAACC
il1b	AJ277166	F3	ATGCCCGAGGGGCTGGGC
		R2	CAGTTGCTGAAGGGAACAGAC
tnfa	AJ413189	FE2	TATGGGGGCATACACAACA
		RE5	TTAAAGTGTCAAACACACCAAA
cox2	AM296029	F1	GAGTACTGGAAGCCGAGCAC
		R1	GATATCACTGCCGCCTGAGT
ccl4	AM765840	F1	GCTGTGTTTGTGCTGATGCT
		R1	GCTGGCTGGTCTTTTGGTAG
tgfb1	AF424703	F	AGAGACGGGCAGTAAAGAA
		R	GCCTGAGGAGACTCTGTTGG
il6	AM749958	F1	AGGCAGGAGTTTGAAGCTGA
		R1	ATGCTGAAGTTGGTGGAAGG
il1r2	AM296027	F	AAGGACTCCAGCTCCACTGA
		R	ACGCCTTCTACATGGACCAC
mmp9	AM905938	F1	GGGGTACCCTCTGTCGATTT
		R1	CCTCCCCAGCAATATTCAGA
mmp13	AM905935	F	CGGTGATTCCTACCCATTTG
		R	TGAGCGGAAAGTGAAGGTCT
timp2a	AM905937	F	CAAAGGTGGTGGGAGAGAAA
		R	TTGACGTCCAGGGTAACTCC
timp2b	AM905936	F	ATGTCGTTATCAGGGCGAAG
		R	AGAAGTGGGAGCGGTGTAGA

2.6. Western blot analysis

The protein concentrations of cell lysates were estimated by the BCA protein assay reagent (Pierce) using bovine serum albumin as a standard. Cell extracts (50 μg per lane) were run in 15% SDS-PAGE at 200 mA and transferred for 50 min at 300 mA to nitrocellulose membranes (BioRad). The blots were developed using a 1/1000 dilution of a rabbit polyclonal antiserum to gilthead seabream interleukin-1β (IL-1β) (Pelegrín et al., 2004), and enhanced chemiluminescence (ECL) reagents (GE Healthcare Life Sciences) according to the manufacturer's protocol. Membranes were then re-probed with a 1:10,000 dilution of a monoclonal anti-β-actin (A5441, Sigma) to confirm a similar protein loading in all lanes.

2.7. Statistical analysis

Data were analysed by two-way analysis of variance (ANOVA) and post hoc multiple treatments comparison analysis, which were performed against control by Dunnett (bilateral) test using the SPSS 13.0 and JMP softwares. In all the cases it was considered $\alpha = 0.05$.

3. Results and discussion

3.1. Collagen and gelatin activate in vitro gilthead seabream professional phagocytes

We firstly evaluated the influence of native and denatured (gelatin) forms of collagen on the respiratory burst of seabream head kidney phagocytes. The results showed that both collagen and gelatin increase the production of ROIs by seabream phagocytes in a dose- and time-dependent manner (Fig. 1A). The stimulatory capacity of the highest concentration of collagen and gelatin used (10 μg/ml) was comparable to that exerted by stimulation with PAMPs (i.e. LPS/VaDNA) at both incubation times. Although, seabream macrophages required at least 5 μg/ml of LPS to be activated *in vitro* (Pelegrín et al., 2004; Sepulcre et al., 2007), we further confirmed that endotoxin contamination of collagen and gelatin was not responsible for the observed effects, since polymyxin B was unable to affect the activation of seabream leukocytes induced by collagen and gelatin (Fig. 1B).

This result prompted us to analyse the gene expression profiles of the two professional phagocytes of this species following stimulation with collagen and gelatin in order to make a direct comparison with LPS/VaDNA-stimulated cells using the same experimental conditions. As regards acidophilic granulocytes, we found that each stimulus resulted in a particular gene expression pattern (Fig. 2). While collagen rapidly (4 h) increased the mRNA levels of the pro-inflammatory cytokine IL-1β, gelatin increased the mRNA levels of IL-1β, chemokine (C–C motif) ligand 4 (CCL4) and MMP9/gelatinase B but with a lower kinetics. In contrast, it was found that LPS/VaDNA resulted in a profound alteration of the immune gene expression pattern. Thus, the expression of the genes coding for the pro-inflammatory molecules IL-1β, cyclooxygenase-2 (COX2), CCL4 and IL-6 as well as of those coding for the ECM remodelling enzymes MMP9 and MMP13 and their tissue inhibitors TIMP2A and TIMP2B significantly increased upon PAMP-stimulation. The stimulation of macrophages with collagen and gelatin also resulted in stimulus-specific gene expression profiles (Fig. 3). Macrophages responded quicker than acidophilic granulocytes to all stimuli, as reported previously for different PAMPs (Sepulcre et al., 2007). Interestingly, while collagen increased the mRNA levels of IL-1β, COX2, CCL4 and to some extent of the anti-inflammatory cytokine TGFβ1, gelatin only increased the mRNA levels of IL-1β in these cells. As expected, LPS/VaDNA drastically increased the mRNA levels of all pro-inflammatory genes analysed but had little effect on the expression of the anti-inflammatory

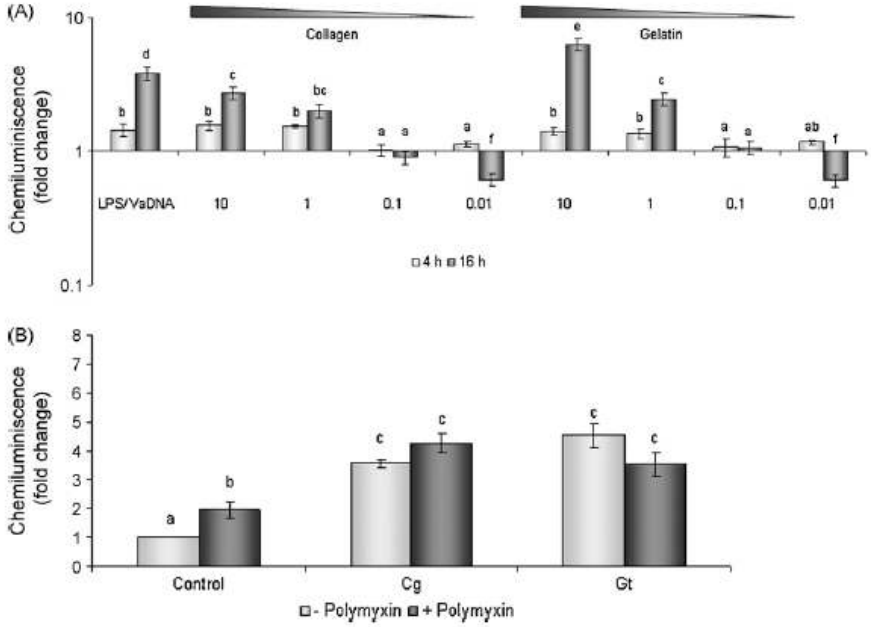

Fig. 1. Collagen and gelatin prime the respiratory burst of gilthead seabream leukocytes. (A) Head kidney leukocytes were incubated for 4 or 16 h with 10 μg/ml LPS and 50 μg/ml VaDNA (positive control) or with 0.01–10 μg/ml collagen or gelatin and their respiratory burst activity was measured as the luminol-dependent chemiluminescence triggered by PMA. Data are presented as mean ± S.E. fold increase relative to cells incubated with medium alone and are representative of 10 independent experiments. (B) Head kidney leukocytes were incubated with 10 μg/ml collagen or gelatin in the absence or presence of 40 μg/ml polymyxin B and their respiratory burst activity was measured as the luminol-dependent chemiluminescence triggered by PMA. Data are presented as mean ± S.E. fold increase relative to cells incubated with medium alone and are representative of four independent experiments. Different letters denote statistically significant differences between the groups according to a Dunnett test. The groups marked with "a" did not show statistically significant differences from control cells.

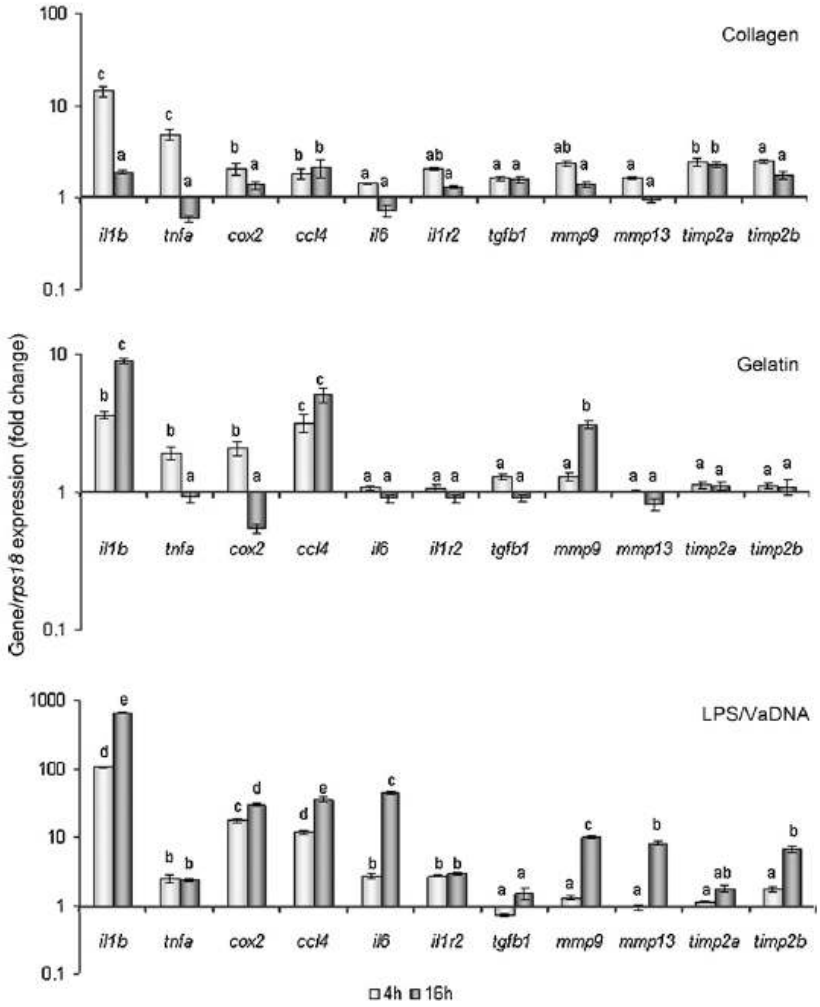

Fig. 2. Collagen and gelatin induce differential gene expression profiles in gilthead seabream acidophilic granulocytes. The mRNA levels of the indicated immune- and ECM remodelling-related genes were determined by real-time RT-PCR in acidophilic granulocytes incubated for the indicated times with 10 μg/ml collagen, 10 μg/ml gelatin or 10 μg/ml LPS and 50 μg/ml VaDNA. Gene expression is normalized against *rps18* and is shown as relative to the mean of non-stimulated cells. Each bar represents the mean ± S.E. of triplicate samples and is representative of three independent experiments. Different letters denote statistically significant differences between the groups according to a Dunnett test. The groups marked with "a" did not show statistically significant differences from control cells.

molecules, TGFβ1 and type II IL-1 receptor (IL-1RII), and the ECM remodelling enzymes, MMPs.

The above results were further confirmed using a specific antibody to gilthead seabream IL-1β (Pelegrín et al., 2004). It was found that the stimulation of head kidney leukocytes with gelatin was able to promote the intracellular accumulation of IL-1β by these cells (Fig. 4A). Similar results were obtained with purified acidophilic granulocytes (Fig. 4B). Collectively, these results indicate that native and denatured collagen is able to induce an activation stage in fish immune cells that differs from that induced by PAMPs, and that it is characterized by the activation of antimicrobial effectors and the production of IL-1β. In fact, the production of IL-1β has been considered a hallmark of the activation of macrophages by collagen upon the engagement of integrins (Pacifici et al., 1991) and DDRs (Matsuyama et al., 2004). It is tempting to speculate, therefore, that fish phagocytes are able to respond to injury through the detection of collagen, and probably other ECM components, as their mammalian counterparts. To ascertain whether the activation by ECM components results in the polarization of macrophages to a phenotype related with the production of factors involved in tissue remodelling and repair (alternatively activated macrophages), further markers need to be developed. However, such a scenario would not be surprising since a recent study has demonstrated that macrophages from the common carp show functional polarization (Joerink et al., 2006).

3.2. Collagen proteolytic fragments mediate the activation of gilthead seabream professional phagocytes

It has been previously shown that collagen fragments produced by digestion with collagenase were more potent than intact collagen in inducing IL-1β release by human blood mononuclear cells (Pacifici et al., 1991). Therefore, we evaluated the effects of a specific inhibitor of gelatinases MMP2 and MMP9 on the respiratory burst of seabream phagocytes induced by collagen as well as the effects of collagen fragments generated by collagenase on this activity. It was found that the MMP2/MMP9 inhibitor V had a dose-dependent inhibitory effect on seabream phagocyte activation by either collagen or gelatin (Fig. 5A). In contrast, this inhibitor exerted a weak effect on the activation of phagocytes by PAMPs (Fig. 5A), which might indicate that seabream leukocytes are able to release collagen upon activation with PAMPs. Released colla-

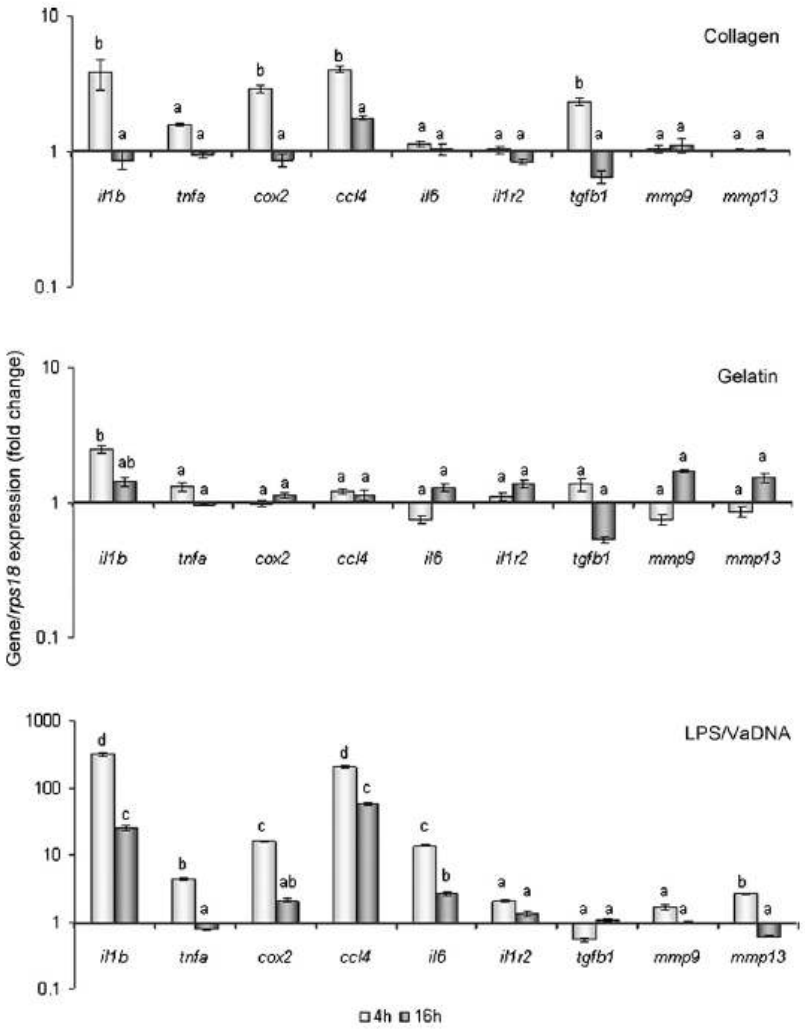

Fig. 3. Collagen and gelatin induce differential gene expression profiles in gilthead seabream macrophages. The mRNA levels of the indicated immune- and ECM remodelling-related genes were determined by real-time RT-PCR in macrophages incubated for the indicated times with 10 μg/ml collagen, 10 μg/ml gelatin or 10 μg/ml LPS and 50 μg/ml VaDNA. Gene expression is normalized against *rps18* and is shown as relative to the mean of non-stimulated cells. Each bar represents the mean ± S.E. of triplicate samples and is representative of three independent experiments. Different letters denote statistically significant differences between the groups according to a Dunnett test. The groups marked with "a" did not show statistically significant differences from control cells.

gen would then be cleavage by MMPs and the resulting proteolytic fragments would in turn activate phagocytes. Interestingly, as the Ki of MMP2/MMP9 inhibitor V for MMP2 and MMP9 is 16 and 180 nM, respectively (Ikejiri et al., 2005), our results also suggest that both enzymes are involved in the solubilization of collagen and/or generation of collagen proteolytic fragments, which in turn are able to activate seabream phagocytes. This is in the agreement with the results obtained by Kubota et al. (2003) that have shown that recombinant Japanese flounder MMP9 showed gelatinolytic activity, but no proteolytic activity against collagen I, which was only solubilized. On the other hand, pre-treatment of collagen and gelatin by collagenase resulted in a higher stimulatory capacity compared to non-treated proteins, while the effect of collagenase on PAMP stimulatory capacity was again weak (Fig. 5B). In addition, the effect of collagenase on collagen was higher than on gelatin, probably reflecting the preference of this enzyme for collagen. All these results, taken together, indicate that proteolytic fragments derived from collagen by the action of different host proteases involved in the remodellation of the ECM, such as MMPs and collagenases, and probably of proteases released by infectious agents, are sensed by fish phagocytes. Therefore, fish phagocytes would not only be able to respond to pathogen-derived molecules (i.e. PAMPs) (Sepulcre et al., 2007) but also to endogenous molecules, such as collagen derived proteolytic fragments, that would signal the presence of damage (recently called damage-associated molecular patterns, DAMPs) (Oppenheim and Yang, 2005). The identification of the receptors involved in the recognition of DAMPs by fish leukocytes will clarify the evolution of the inflammatory response in vertebrates. In fact, although most DAMPs identified so far are known to signal through TLR4/MD2/CD14 receptor complex in mammals, including hyaluronan oligomers, fibrinogen, heparan sulfate, β-defensin 2, biglycan, heat shock proteins and high mobility group box 1 (HMGB1) (reviewed by Mollen et al., 2006), MD2 and CD14 are absent in fish (Iliev et al., 2005) and the TLR4 has only been identified in the zebrafish (*Danio rerio*) and is clearly absent from most evolutionary advanced fish, such as the Japanese pufferfish (*Takifugu rubripes*) and the spotted green pufferfish (*Tetraodon nigroviridis*) (Roach et al., 2005). In addition,

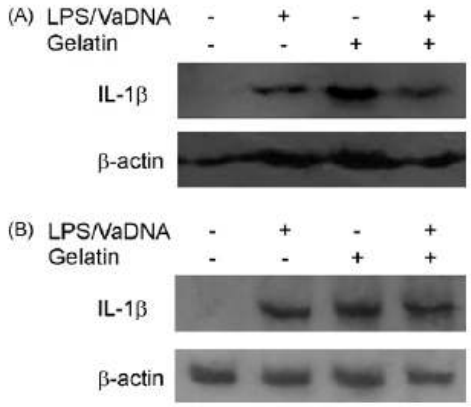

Fig. 4. Gelatin induces the accumulation of IL-1β in gilthead seabream leukocytes. Head kidney leukocytes (A) and purified acidophilic granulocytes (B) were stimulated overnight with 10 μg/ml LPS and 50 μg/ml VaDNA and/or 10 μg/ml and/or gelatin. Whole cell lysates (5×10^6 cells) were probed with antibodies to seabream IL-1β and β-actin. The results are representative of three independent experiments.

we have recently shown that the two zebrafish TLR4 orthologues are not involved in the recognition of enterobacterial LPS (Sepulcre et al., 2009), the cognate and best characterized ligand for the TLR4 complex of mammals. In any case, we have shown for the first time that the innate immune system of fish is able to respond to tissue injury together to the well-established response to infection.

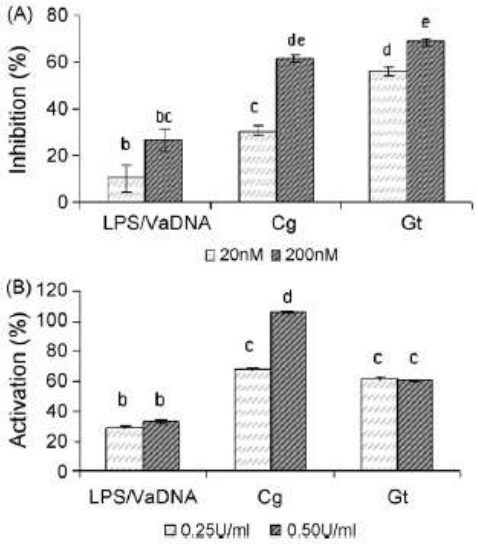

Fig. 5. Collagen proteolytic fragments mediate the activation of gilthead seabream leukocytes (A). Head kidney leukocytes were incubated for 16 h with 10 μg/ml LPS and 50 μg/ml VaDNA, 10 μg/ml collagen (Cg) or 10 μg/ml gelatin (Gt) in the absence or presence of 20–200 nM MMP2/MMP9 inhibitor V and their respiratory burst activity was measured as the luminol-dependent chemiluminescence triggered by PMA. Data are presented as the percentage of inhibition relative to cells incubated without inhibitor. (B) Six-well culture plates were coated overnight with 10 μg/ml LPS and 50 μg/ml VaDNA, 10 μg/ml collagen or 10 μg/ml gelatin and then treated for 10 min with 0.25–0.50 U/ml collagenase at 37 °C. The enzyme was removed with PBS, the cells were then seeded and incubated for 16 h before their respiratory burst activity was measured as the luminol-dependent chemiluminescence triggered by PMA. Data are presented as the percentage of activation relative to non-stimulated cells. For both figures, each bar represents the mean ± S.E. of triplicate samples and is representative of four independent experiments. Different letters denote statistically significant differences between the groups according to a Dunnett test.

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2. CORRELATED EXPRESSION PROFILE OF EXTRACELLULAR MATRIX-RELATED MOLECULES DURING THE INFLAMMATORY RESPONSE OF THE TELEOST FISH GILTHEAD SEABREAM

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Correlated expression profile of extracellular matrix-related molecules during the inflammatory response of the teleost fish gilthead seabream

Patricia Castillo-Briceño[a], Marta Arizcun-Arizcun[b], José Meseguer[a], Victoriano Mulero[a], Alfonsa García-Ayala[a,*]

[a] Department of Cell Biology and Histology, University of Murcia, Murcia 30100, Spain
[b] Oceanographic Centre of Murcia, Spanish Oceanographic Institute (IEO), Puerto de Mazarrón, 30860 Murcia, Spain

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ABSTRACT

Extracellular matrix (ECM) components, in addition to their structural functions, interact with cell surface receptors and intracellular components to modulate the transduction of signals for cell growth, differentiation, migration, proliferation, polarization, apoptosis and inflammation. Our previous findings in the gilthead seabream (*Sparus aurata* L.), a marine seasonal hermaphrodite teleost fish, have shown that both endocrine and immune stimuli modulate the expression of matrix metalloproteases (MMPs) and tissue inhibitors of MMP (TIMPs). In addition, collagen type I (COL1) induces the expression of some pro-inflammatory cytokines and MMPs in professional phagocytes. Consequently, in this study we use real-time RT-PCR to analyze the gene expression profile of several ECM-related molecules (MMP-2, -9 and -13, TIMP-2a, and -2b, COL1A1, and integrin β1a) in different organs of adult specimens as well as in response to innate immune challenges. Our results showed that liver had the lowest basal levels of them, although they were clearly modulated during injury and infection. In the same way, ECM-related molecules seem to participate in pro-inflammatory processes, being of particular interest COL1 which is synthesized by immune cells and is able to act as autocrine/paracrine stimulus for them. Lastly, we propose that the observed correlations between ECM-related molecules during the inflammatory response should be considered to obtain a more accurate picture of their roles in this process.

1. Introduction

The structural distinctiveness of the properties of tissues and organs is considered to be determined by the extracellular matrix (ECM) and the cells that produce it (Tsang et al., 2009). However, it is only since the discovery of integrins and other ECM receptors two decades ago that ECM has become interesting for researchers, not only as an intercellular and tissue scaffold, but also as a complex extracellular environment and key element in a variety of cellular processes covering a wide range of functionality (Huxley-Jones et al., 2009; Rozario and DeSimone, 2009). ECM interacts with diverse cell components and modulates the transduction of signals that regulate cell growth, differentiation, migration, proliferation, polarization, death and inflammation (Hynes, 2009; Tsang et al., 2009). Moreover, the correct expression and function of genes controlling ECM–cell and cell–cell interactions provide the necessary environment for normal multicellular tissues behavior (Werb, 1997).

Collagen (COL) molecules are a structurally and developmentally complex family of proteins, which constitute the major components of the ECM of all metazoans and appear to be relevant during their evolution (Heino et al., 2009; Hynes, 2009). They have diverse functions and play a dominant role in maintaining tissue structure (Canty and Kadler, 2005; Myllyharju and Kivirikko, 2004). There are almost 30 COL types, among them COL type I accounts for most of the collagen mass in non-cartilaginous tissues, normally as heterotypic fibrils (Canty and Kadler, 2005; Wenstrup et al., 2004; Wess, 2005). Fibroblasts, monocytes, endothelial and other cell types are able to efficiently secrete proCOLs *in vivo* (Wess, 2005), and these are processed and assembled in the cell surface (Canty and Kadler, 2005). The interactions between cells and COL in the ECM are mediated by a variety of widely described receptors p.e. integrins (ITGs), which upon COL binding, may activate other molecules, such as matrikines, matrix metalloproteases (MMPs), tissue inhibitor of MMP (TIMPs), cytokines and growth factors, which are indispensable for remodeling, inflammatory, immune or wound healing responses (Garnotel et al., 2000; Heino, 2000; Herr and Farndale, 2009; Lee et al., 2007; Leitinger and Hohenester,

Abbreviations: AGs, acidophilic granulocytes; Cq, quantification cycle; EE2, 17α-ethynylestradiol; MCs, macrophages; VaDNA, genomic DNA from *Vibrio anguillarum*.

* Corresponding author. Tel.: +34 868884968; fax: +34 868883963.
E-mail address: agayala@um.es (A. García-Ayala).

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2007; Myllyharju and Kivirikko, 2004; Schultz and Wysocki, 2009; Tran et al., 2004; Vogel, 2001).

ITGs have been widely described as the main cell surface receptors for several eukaryotic organisms (Delon and Brown, 2007; Hynes, 2002). This is a superfamily of αβ heterodimers involved not only in numerous cellular processes including leukocyte traffic, immune response, tissue repair and remodeling, but also in autoimmune diseases and cancer (Delon and Brown, 2007; Hynes, 2002). Four of the β1 integrins (α1β1, α2β1, α10β1 and α11β1) act primarily as COL receptors and contain an I-domain (also called A, for its homology in von Willebrand factor A domain), that is able to bind directly to COL, but which is only present in vertebrates (Huhtala et al., 2005; Johnson et al., 2009; Leitinger and Hohenester, 2007; Takada et al., 2007). COL–ITG binding could play a control role during wound healing, particularly for MMP release and cellular adhesion (Mócsai et al., 2006; Pilcher et al., 1999; Stefanidakis and Koivunen, 2006; Zweers et al., 2007).

MMPs are zinc-dependent endopeptidases involved in extracellular proteolysis, which is essential in both physiological and pathological processes. Present interest in MMPs is due to their involvement in several human diseases, although less attention has been paid to their substrate repertoires and involvement in physiological pathways (Butler and Overall, 2009; Stefanidakis and Koivunen, 2006). In that sense, MMP-2, -9 and -13 are known to act synergistically on inflammation, vasculature recruitment, ECM modulation, bone turnover and chemotaxis (Amălinei et al., 2007; Butler and Overall, 2009; Stevens et al., 2009; Takaishi et al., 2008).

MMP activity is closely regulated by TIMPs to maintain a balance in tissue remodeling and degradation in the ECM. In vertebrates, TIMPs have evolved from a common ancestor through the duplication of pre-existing genes (Huxley-Jones et al., 2007). In the teleost *Danio rerio* a TIMP-1 ortholog is absent, but there are representatives of the other three classes, including duplicates of TIMP-2 (Wyatt et al., 2009). In mammals, it is described that an imbalance between MMPs and TIMPs expression is characteristic in various medical conditions, such as cancer, sepsis, arthritis, tissue ulcers and fibrosis (Amălinei et al., 2007; Chirco et al., 2006; Ljumovic et al., 2004; Sternlicht and Werb, 2001). Therefore, the MMP/TIMP ratios and its expression levels have been suggested as disease biomarkers (Del Casar et al., 2009; Lorente et al., 2009). However, these parameters are not always considered a valid measure in pathophysiologic conditions like endometriosis, in which the correlation between MMPs and their inhibitors is more effective as indicator (Salata et al., 2008). As well, in myocardium from patients with sudden unexpected cardiac death, correlation of MMP-9 and other ECM-related molecules with troponin (a muscle contraction protein) was useful to characterize that condition (Ceauşu et al., 2009).

The gilthead seabream (*Sparus aurata* L.) is a seasonal marine teleost hermaphrodite species with a bi-sexual gonad, which offers an interesting model for inflammatory response studies. Previously we observed that TIMP-2a and -2b are expressed more strongly during the testicular remodeling stage, which is characterized by an infiltration of acidophilic granulocytes (AGs), the main professional phagocytic cell type in this species (Chaves-Pozo et al., 2008), and also this TIMPs are induced during the inflammatory response of professional phagocytes to danger and pathogen associated molecular patterns (DAMPs and PAMPs, respectively) (Castillo-Briceño et al., 2009). Our previous findings concerning the modulation of MMP and TIMP expression in gilthead seabream in inflammatory conditions (Castillo-Briceño et al., 2009; Chaves-Pozo et al., 2008) also suggested an underlying correlation on the mRNA levels of these molecules, which may be modified by DAMPs and PAMPs (Castillo-Briceño et al., 2009). Therefore, this correlated expression of the ECM-related molecules seemed to merit closer analysis as regards the possible role of a correlated modulation as part of the innate immune response.

2. Materials and methods

2.1. Animals

Healthy specimens of gilthead seabream (*Sparus aurata* L., Actinopterygii, Teleostei) were obtained from natural spawning of a captive broodstock in the hatchery facilities of the Spanish Oceanographic Institute (IEO) of Murcia using the "green water" larval culture technique and following their previously described general protocols for adult fish culture (Castillo-Briceño et al., 2009). Animals were fasted for 24 h before sampling. The experiments performed comply with the Guidelines of the European Union Council (86/609/EU) and the Bioethical Committee of the University of Murcia (Spain) for the use of laboratory animals.

2.2. Organ sampling

Adult specimens (1150 g mean weight) were bled and dissected, and the organs were collected according to their relevant immune (gills, thymus, spleen, liver, head kidney, midgut, peritoneal exudate and blood), endocrine (brain, pituitary, testis and ovary), physiological (kidney and heart-ventricle) or structural (muscle, skin and caudal fin) functions. In all cases, the samples were maintained in TRIzol Reagent (Invitrogen) at −80 °C until they were processed for RNA extraction.

2.3. Cell isolation, culture and treatments

AGs isolated by magnetic-activated cell sorting (MACS) (Sepulcre et al., 2002) and macrophages (MCs) monolayers (Roca et al., 2006) were obtained from head kidney, and cultured in RPMI-1640 (Gibco) adjusted to gilthead seabream serum osmolarity (353.33 mosmol) with 0.35% NaCl (sRPMI) and supplemented with 1% fetal bovine serum (FBS) (Invitrogen), 100 IU/ml penicillin, and 100 μg/ml streptomycin (P/S, Biochrom), as described elsewhere (Castillo-Briceño et al., 2009; Roca et al., 2008). Endothelial cells (EECs) were isolated from endocardium as previously described (Koren et al., 1997; Roca et al., 2008), with slight modifications. Cells were seeded on 75-cm^2 primary tissue culture plastic flasks, pre-coated with 0.1% gelatin in PBS for 30 min at room temperature, in sRPMI supplemented as for AGs and MCs, plus 2 μg/ml amphotericin B (Biochrom). After 48 h of incubation at 23 °C, adherent EECs were washed twice with sRPMI supplemented with 5% FBS and 50 μg/ml gentamicin and left to grow in this medium. AGs, MCs and EECs were incubated with 10 μg/ml collagen type I from calf skin (Sigma) or 50 μg/ml genomic DNA of *Vibrio anguillarum* (VaDNA), as previously described (Castillo-Briceño et al., 2009). Control and treated cells were collected after 3 h of incubation and lysed in TRIzol.

2.4. Experimental infections

Adult fish (150 g mean weight) were infected with *V. anguillarum* R82 cells (serogroup 01) by intraperitoneal injection according to the protocols normally used in our laboratory (López-Castejón et al., 2007). Four hours after the injection, the immune competent organs and components (gills, thymus, spleen, liver, head kidney, peritoneal exudate and blood) from control and infected animals were sampled and maintained in TRIzol as previously stated.

2.5. Induction of liver injury by 17α-ethynylestradiol

Adult fish (170 g mean weight) were exposed to 17α-ethynylestradiol (EE2) by oral ingestion, according to Angus et al.

Table 1
Primer sequences used for gene expression analysis. The gene symbols followed the Zebrafish Nomenclature Guidelines (http://zfin.org/zfinfo/nomen.html).

Gene	Accession number	Primer name	Sequence (5′ → 3′)
rps18	AM490061	F	AGGGTGTTGGCAGACGTTAC
		R	CTTCTGCCTGTTGAGGAACC
mmp2	FN649419	F1	ACTATGACCGCGACAAGTCC
		R1	GTAACCTTGGTCGGGACAGA
mmp9	AM905938	F1	GGGGTACCCTCTGTCGATTT
		R1	CCTCCCCAGCAATATTCAGA
mmp13	AM905935	F	CGGTGATTCCTACCCATTTG
		R	TGAGCGGAAAGTGAAGGTCT
timp2a	AM905937	F	CAAAGGTGGTGGGAGAGAAA
		R	TTGACGTCCAGGGTAACTCC
timp2b	AM905936	F	ATGTCGTTATCAGGGCGAAG
		R	AGAAGTGGGAGCGGTGTAGA
col1a1	DQ324363	F2	GCTCTCAGCCAGAGGATGTC
		R2	TGTAGGCGATGCTGTTCTTG
itgb1a	FN649420	F2	AAGGGAGACGAGTTCAATCGGT
		R1	CACCAGCAGACGAGTCACAT

(2005). The food for the animal treatments was supplemented with an EE2 solution in ethanol (50 μg/g of food), while only ethanol was added to control food. Both feeds were allowed to dry and then stored at 4 °C. Animals were fed normally and liver was sampled at 28 days, when external liver hyperplasia was obvious.

2.6. Analysis of gene expression

Samples in TRIzol were thawed on ice and processed following the manufacturer's instructions for RNA extraction. To avoid DNA contamination, RNA suspended in water was treated with DNase I (1 U/μg RNA) Amplification grade (Invitrogen). The RT-qPCR was performed using a SuperScript III ReverseTranscriptase Kit (Invitrogen) to synthesize first strand cDNA from 1 μg of total RNA, and SYBR Green PCR Core Reagents (Applied Biosystems) with an ABI PRISM 7500 instrument (Applied Biosystems) according to the respective manuals and as previously described (Castillo-Briceño et al., 2009). For each mRNA, gene expression was normalized to the ribosomal protein S18 content of each sample using the comparative Cq method ($2^{-\Delta\Delta Cq}$) (Bustin et al., 2009). The primers used are shown in Table 1. In all cases, each PCR was performed in triplicate and repeated with at least two independent samples.

2.7. Statistical analysis

Data for the whole organs sampled were analyzed by a one-way analysis of variance (ANOVA) and Tukey's multiple comparisons test, for each gene. Variations in EE2 induced inflammation of the liver and in organs after *V. anguillarum* infection were analyzed by a two-way ANOVA and Bonferroni's post-test compared with the controls. The mRNA levels in MC, AGs and EECs, and for stimulated AGs were analyzed by two-way ANOVA and Bonferroni's post-tests comparing each type or condition with all the others. Correlation between ECM-related molecules in AGs was analyzed by Pearson's matrix correlation test (comparing each pair of data sets). The analyses performed were the most restrictive, following the recommendations of the statistical software used (Prism 5 for Windows version 5.00) depending on the data set characteristics, to detect the greatest significant differences among groups at $p < 0.05$ and considering the variability in the expression levels between the analyzed genes.

3. Results

3.1. ECM-related molecules show differential expression profiles in individual organs

The mRNA analysis of different organs and tissues in adult specimens showed that the MMPs, TIMPs, COL1A1 and ITGB1a present particular profiles (Fig. 1). In general, caudal fin showed high expression levels of all the genes analyzed, except TIMP-2b, while liver was always in the group showing the lowest expression. Of note was the fact that COL1A1 and TIMP-2b were expressed in a significantly higher degree than the other genes. In addition, the expression of TIMP-2b was mainly restricted to the immune tissues (gills, thymus, head kidney, blood, peritoneal exudate and, to some extent, the spleen) and showed much higher levels in peritoneal exudate and blood. Similarly, the expression of MMP-13 was also restricted to the immune tissues, with the exception of the caudal fin.

3.2. The expression of TIMP-2b is induced in immune competent organs after Vibrio anguillarum infection

After *V. anguillarum* infection of adult specimens, there was a generalized robust increase in the mRNA levels of TIMP-2b in immune competent organs (gills, thymus, spleen, liver and head kidney) and tissues (peritoneal exudate and blood) (Fig. 2). In contrast, TIMP-2a presented a modest increase in liver and blood. MMP-13 expression was inhibited in spleen and gills, as MMP-2 and COL1A1 expression in gills. While, the expression levels of MMP-9 and ITGB1a were not significantly modulated in any organ upon infection

3.3. The expression of ECM-related molecules is induced in the liver of fish fed with 17α-ethynylestradiol

EE2 was used to induce hepatic injury since this xenoestrogen is known to cause toxicity in fish, particularly in the liver and kidney (Lynn et al., 2003; Metcalfe et al., 2001). Hyperplasia of the liver was observed in fish fed with EE2 for 28 days accompanied by a significant increase in the expression of all the ECM-related molecules analyzed: MMP-2, MMP-9, MMP-13, TIMP-2a, TIMP-2b, COL1A1 and ITGB1a (Fig. 3).

3.4. ECM-related molecules show differential expression profiles in cells involved in the inflammatory response

In general terms, the ECM-related molecules in AGs and MCs had a slightly different expression profile from the observed in EECs (Fig. 4). AGs and MCs showed higher expression levels of MMP-9 and TIMP-2a but lower COL1A1 levels. Moreover, AGs also had higher levels of MMP-13 and TIMP-2b than the other cell types, while MCs showed the highest expression levels of MMP-2. Finally, the expression of ITGB1a was similar in all the groups.

3.5. Correlation of the expression of ECM-related molecules is modulated in acidophilic granulocytes following VaDNA and COL1 stimulation

Stimulation of AGs with VaDNA for 3 h significantly induced the expression of TIMP-2a (2.17 ± 0.26), TIMP-2b (2.98 ± 0.35) and COL1A1 (29.17 ± 3.44), whereas that stimulated with COL1 presented non-significant changes (Fig. 5). However, the correlation among the genes in both conditions presented different pictures from the control (Fig. 6). Thus, in non-stimulated cells, TIMP-2b was correlated with all the ECM-related molecules considered (Pearson

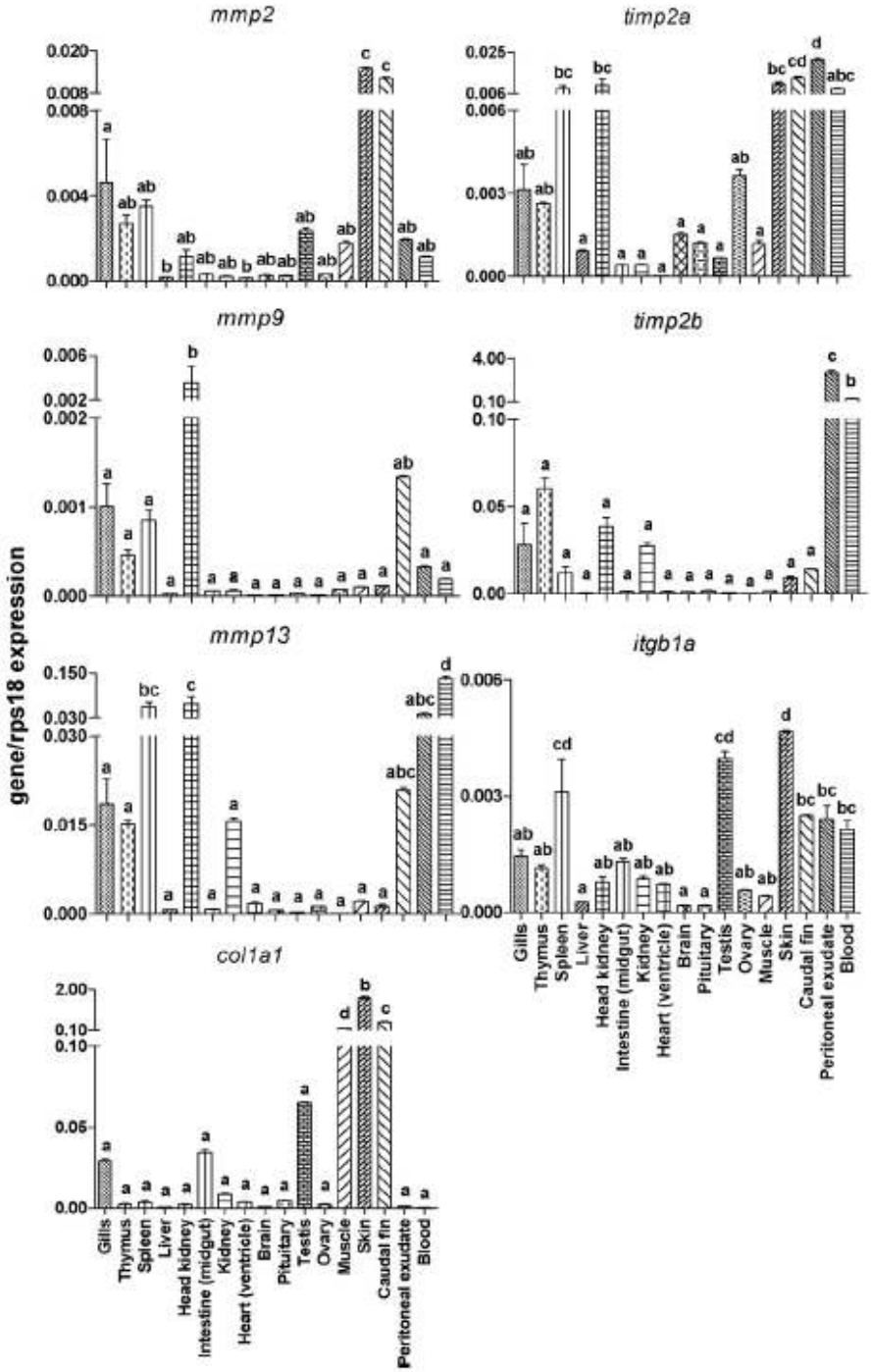

Fig. 1. ECM-related molecules have differential gene expression profiles in organs of adult *Sparus aurata* L. specimens. The mRNA levels of the indicated ECM-related genes were determined by RT-qPCR in amplification products obtained from pools of whole organs of at least eight fish. Gene expression is standardized against rps18. Each bar represents the mean ± S.E. of triplicate measures. Different letters denote statistically significant differences between groups according to Tukey's test from a one-way ANOVA ($p < 0.05$).

$r > 0.64$, $p < 0.05$), except COL1A1, while ITGB1a was also correlated with all genes analyzed (Pearson $r > 0.79$, $p < 0.05$), except MMP-9 (Table 2). In contrast, after VaDNA stimulation MMP-2, TIMP-2a and TIMP-2b were strongly correlated with all genes (Pearson $r > 0.90$, $p \leq 0.001$), except MMP-13 (Table 3), while upon COL1 stimulation only MMP-2 was correlated with all genes (Pearson $r > 0.75$, $p < 0.05$), except MMP-13 (Table 4). As regards the ECM-related molecules: (i) MMP-13 correlated with both TIMPs and ITGB1a in non-stimulated cells (Table 2), while it did not show any significant correlation with any other molecules in stimulated cells (Tables 3 and 4); (ii) MMP-9 was found to correlate with COL1A1 (Pearson $r > 0.80$, $p < 0.01$) in non-stimulated cells but not

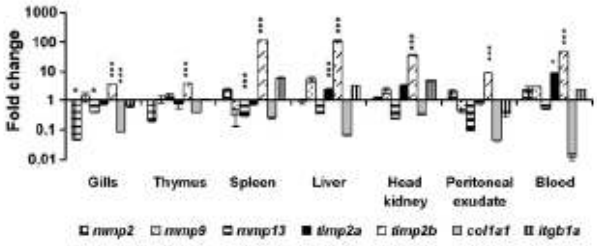

Fig. 2. ECM-related molecules show differential gene expression profile modulations in immune competent organs of *Sparus aurata* L. during *Vibrio angullarum* experimental infection *in vivo*. The mRNA levels of the indicated ECM-related genes were determined by RT-qPCR in amplification products obtained from pools of whole organs of control and infected adult fish (six individuals for each treatment). Gene expression is standardized against rps18 and relative to control fish. Each bar represents the mean ± S.E. of triplicate measures. The symbol "*" denotes statistically significant differences from the control, according to Bonferroni's post-test for a two-way ANOVA ($p < 0.05$).

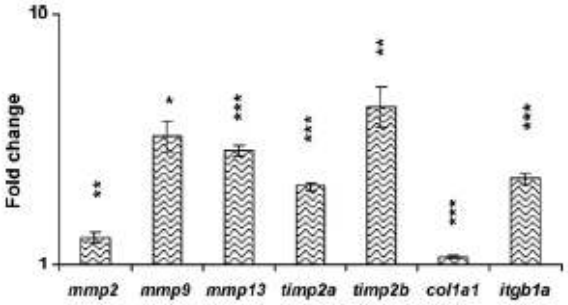

Fig. 3. The gene expression of ECM-related molecules increased during 17α-ethynylestradiol (EE2)-induced inflammation of liver of *Sparus aurata* L. The mRNA levels of the indicated ECM-related genes were determined by RT-qPCR in amplification products obtained from pools of whole livers of control and EE2-treated adult fish (six individuals for each condition). Gene expression is standardized against rps18 and relative to control fish. Each bar represents the mean ± S.E. of triplicate measures. The symbol "*" denotes statistically significant differences from the control according to Bonferroni's post-test for a two-way ANOVA ($p < 0.05$).

in VaDNA- (Table 3) and COL1-stimulated (Table 4) cells, and (iii) COL1A1 correlation with ITGB1a in non-stimulated cells (Pearson $r > 0.82$, $p = 0.001$) increased upon stimulation with VaDNA (Table 3) and COL1 (Table 4).

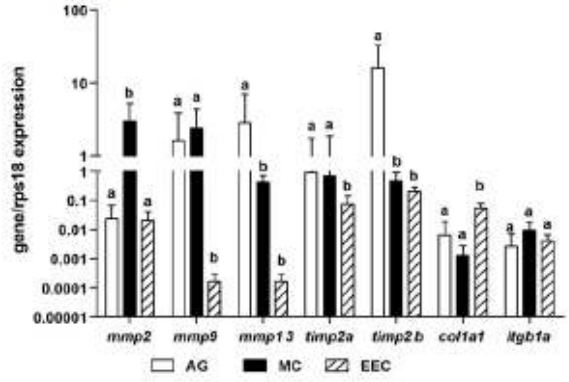

Fig. 4. ECM-related molecules show differential gene expression profiles in wound healing-related cell types of *Sparus aurata* L. The mRNA levels of the indicated ECM-related genes were determined by RT-qPCR in amplification products obtained from isolated fractions of acidophilic granulocytes (AG), macrophages (MC) and endothelial cells (EEC) of at least eleven adult fish. Gene expression is standardized against rps18. Each bar represents the mean ± S.E. of triplicate measures. Different letters denote statistically significant differences between the groups according to Bonferroni's post-test for a two-way ANOVA ($p < 0.05$).

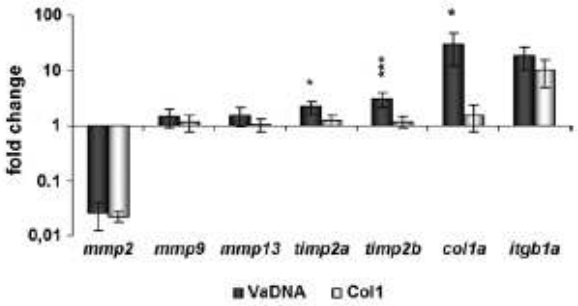

Fig. 5. *Vibrio angullarum* genomic DNA (VaDNA) altered the gene expression profile of some ECM-related molecules in acidophilic granulocytes (AGs) of *Sparus aurata* L. The mRNA levels of the indicated ECM-genes were determined by RT-qPCR in amplification products obtained from AGs incubated for 3 h with 10 μg/ml collagen type I (COL1) or 50 μg/ml VaDNA. Gene expression is standardized against rps18 and relative to control cells. Each point represents the mean ± S.E of at least six fish. Statistically significant differences against control were marked by "*" according to Bonferroni's post-test for a two-way ANOVA ($p < 0.05$).

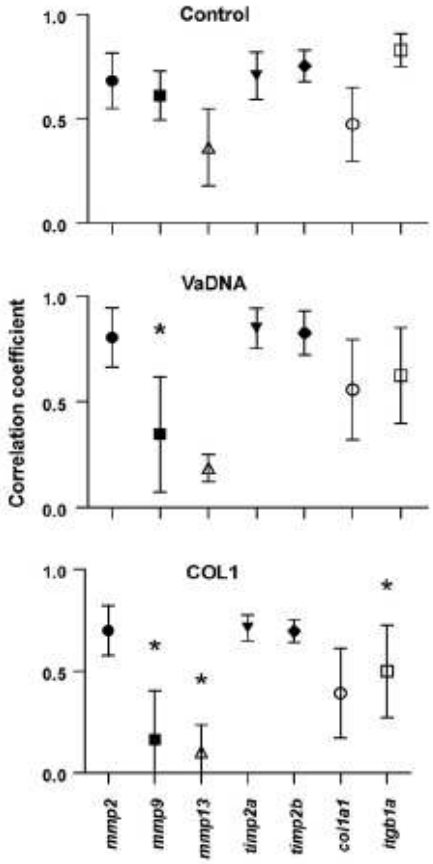

Fig. 6. Collagen type I (COL1) changes the gene expression correlation profile of ECM-related molecules in acidophilic granulocytes (AGs) of *Sparus aurata* L. The mRNA levels of the indicated ECM-genes were determined by RT-qPCR in amplification products obtained from AGs incubated for 3 h with 10 μg/ml COL1 or 50 μg/ml *Vibrio angullarum* genomic DNA (VaDNA). Gene expression is standardized against rps18 and representative of triplicate measures and six independent experiments. Each point represents the mean ± S.E. of correlation coefficients for each gene against all the others based in a matrix correlation test (perfect correlation = 1). Statistically significant differences against control were marked by "*", between COL1 and VaDNA were not significant differences according to Bonferroni's post-test for a two-way ANOVA ($p < 0.05$).

Table 2
Correlation of ECM-related molecule gene expression in head kidney acidophilic granulocytes of *Sparus aurata* L. specimens after 3 h of culture in normal medium.

	mmp2	*mmp9*	*mmp13*	*timp2a*	*timp2b*	*col1a1*	*itgb1a*
mmp2		**0.89***	**0.08**	**0.60**	**0.76***	**0.78***	**0.99***
mmp9	0.001		**0.11**	**0.61***	**0.78***	**0.80***	**0.47**
mmp13	0.849	0.705		**0.83***	**0.64***	**−0.27**	**0.79***
timp2a	0.087	0.015	<0.001		**0.96***	**0.24**	**0.98***
timp2b	0.018	0.003	0.023	<0.001		**0.45**	**0.92***
col1a1	0.002	0.009	0.484	0.531	0.222		**0.82***
itgb1a	<0.001	0.202	0.012	<0.001	<0.001	0.001	
Total correlated genes	4	4	3	4	5	3	5

r = correlation coefficient (bold style numbers).
p = *p* value (two-tailed).
* Significant *r* ($p > 0.05$).

Table 3
Correlation of ECM-related molecule gene expression in head kidney acidophilic granulocytes of *Sparus aurata* L. specimens after 3 h of incubation with *Vibrio anguillarum* genomic DNA.

	mmp2	*mmp9*	*mmp13*	*timp2a*	*timp2b*	*col1a1*	*itgb1a*
mmp2		**0.99***	**0.12**	**0.94***	**0.91***	**0.90***	**0.97***
mmp9	<0.001		**0.01**	**0.92***	**0.91***	**−0.38**	**−0.37**
mmp13	0.769	0.985		**0.38**	**0.31**	**0.03**	**0.28**
timp2a	<0.001	<0.001	0.316		**0.98***	**0.89***	**0.96***
timp2b	0.001	0.001	0.419	<0.001		**0.92***	**0.92***
col1a1	0.001	0.229	0.935	0.001	<0.001		**0.97***
itgb1a	<0.001	0.237	0.458	<0.001	<0.001	<0.001	
Total correlated genes	5	3	0	5	5	4	4

r = correlation coefficient (bold style numbers).
p = *p* value (two-tailed).
* Significant *r* ($p > 0.05$).

4. Discussion

The tightly coordinated regulation of ECM-related molecules in gilthead seabream would agree with the general notion of a finely tuned balance of MMPs/TIMPs in the activation of immune pathways (Stevens et al., 2009; Werb, 1997). The differential mRNA expression pattern of the ECM-related molecules in organs of adult specimens suggests their possible role in tissue remodeling and regeneration. Thus, the high expression of these molecules in caudal fin reflects its regenerative potential (Yin and Poss, 2008), while the prominent expression of MMP-9 and -13 in head kidney, and other immune competent organs, confirms their contribution to the innate immune response in the gilthead seabream (Castillo-Briceño et al., 2009; Chaves-Pozo et al., 2008) and other vertebrates (Butler and Overall, 2009). Interestingly, MMP-9 appears to play a less important role in the spleen, while ITGB1a could play an immune modulation role similar to mammalian ITGBs (Huhtala et al., 2005; Johnson et al., 2009; Mócsai et al., 2006; Takada et al., 2007). The strong expression of COL1A1 in muscle, skin, caudal fin and testis would agree with its involvement in structural tissue configuration (Herr and Farndale, 2009; Huxley-Jones et al., 2009; Wenstrup et al., 2004; Wess, 2005). Similarly, the ubiquitous expression of TIMP-2a hints at a generalized regulatory function probably equivalent to the multifaceted role of TIMP-1 in mammals (Bednarek et al., 2009; Chirco et al., 2006; Stefanidakis and Koivunen, 2006). Its strong expression in peritoneal exudate and blood further supports a generalized regulatory function for TIMP-2a, since circulating cells might represent an additional source of this inhibitor for all organs. In sharp contrast, TIMP-2b might play a specific role in the immune response since its expression is largely restricted to immune competent organs and it is the only strongly induced upon bacterial infection in these organs.

The MMP and TIMP expression profile in AGs and MCs suggest that these ECM-related molecules facilitate their infiltration into wound, as occurs in mammals during tissue restoration (Pilcher et al., 1999; Schultz and Wysocki, 2009; Tran et al., 2004; Zweers et al., 2007). On the other hand, the higher COL1A1 levels observed in EECs agree with their expected role in COL secretion and deposition for structure maintenance (Canty and Kadler, 2005; Lindblad, 1998).

Despite the low levels of expression of ECM-related molecules in the liver of adult specimens of gilthead seabream, in the face of

Table 4
Correlation of ECM-related molecule gene expression in head kidney acidophilic granulocytes of *Sparus aurata* L. specimens after 3 h of incubation with collagen type I.

	mmp2	*mmp9*	*mmp13*	*timp2a*	*timp2b*	*col1a1*	*itgb1a*
mmp2		**0.82***	**0.09**	**0.78***	**0.85***	**0.75***	**0.91***
mmp9	0.007		**−0.30**	**0.71***	**0.57**	**−0.39**	**−0.43**
mmp13	0.812	0.433		**0.43**	**0.52**	**−0.21**	**0.06**
timp2a	0.013	0.032	0.245		**0.85***	**0.65**	**0.84***
timp2b	0.004	0.109	0.146	0.004		**0.66**	**0.73***
col1a1	0.020	0.214	0.593	0.056	0.055		**0.89***
itgb1a	0.001	0.161	0.887	0.004	0.024	<0.001	
Total correlated genes	5	2	0	4	3	2	4

r = correlation coefficient (bold style numbers).
p = *p* value (two-tailed).
* Significant *r* ($p > 0.05$).

bacterial infection the expression of these molecules was similar to the levels detected in other immune competent organs, confirming their relevance in the hepatic response to diseases (Ljumovic et al., 2004). More interestingly, the simultaneous induction of MMPs, TIMPs, COL1A1 and ITGB1a upon steroid-induced injury largely mirrors the upregulation of ECM-related molecules described for common fibrosis in liver diseases in mammals (Ljumovic et al., 2004). Additionally, ITGB1a expression is regulated by EE2, similarly to the novo synthesis and activation of ITGB1 induced by estrogens in mammals (Bowen and Hunt, 2000).

After bacterial infection, MMP and TIMP expression profiles point to the prevalence of MMPs inhibition by endogenous inhibitors, similar to the situation found in human sepsis where increased TIMP-1 expression levels lead to a diminished MMP-9/TIMP-1 ratio in plasma (Lorente et al., 2009). Of note is the stronger inhibition of MMP-2 and COL1A1 in gills, probably as part of a mechanism to prevent new infections, coinciding with an increase in IL-1β and IL-1RII (López-Castejón et al., 2007) and probably related to phagocytic activity, which has been proposed to act as a second line of defense (Mulero et al., 2007).

Our results concerning stimulated AGs also point to a key role for ECM-related molecules in inflammation and immunity. Gilthead seabream AGs have been described as professional phagocytes and share many functions with mammalian neutrophils, although they also play non-immune functions, such as the regulation of gonad activity (Chaves-Pozo et al., 2008). COL1 stimulation of AGs affected their correlation profile of the ECM-related molecules, similarly to the effect of VaDNA stimulation, and both differed from the controls. That suggests a tightly coordinated regulation in the expression of these molecules during inflammation and also supports our previous proposal about COL1 as a DAMP in this species (Castillo-Briceño et al., 2009). Since the variation in mRNA levels of the ECM-related molecules was non-significant in COL1-stimulated AGs (probably due to a weak effect and a high variability between animals), it is suggested that the correlation profile is a more sensitive parameter to detect differences in ECM-related molecules than the level changes or MMP/TIMP ratios, which are generally considered as the markers for pathological conditions and misbalance indicators (Chirco et al., 2006; Del Casar et al., 2009; Sternlicht and Werb, 2001).

The increased mRNA levels of TIMPs and COL1A1 in AGs after PAMP stimulation (VaDNA) pointed to the participation of these cells in ECM integrity maintenance through the regulation of MMP activity and COL deposition. The lack of correlation of MMP-13 with any other ECM-related molecule in activated AGs strongly suggests a coordinated action of MMPs, as described in mammals, where MMP-13 inhibition results in COL accumulation (Butler and Overall, 2009; Takaishi et al., 2008) and its enzymatic activity precedes the degradation of COL by MMP-2 and -9 (Takaishi et al., 2008). Thus, the COL produced by activated AGs might also auto- and paracrinally activate these phagocytes to synthesize pro-inflammatory molecules (Castillo-Briceño et al., 2009). A similar process has been found in mammalian monocytes, which participate in ECM reconstitution in wound environments (Lindblad, 1998) and subsequent differentiation in MCs, which produce pro-inflammatory molecules in the presence of COL1 by ITGB1 binding regulation (De Fougerolles and Koteliansky, 2002). It is tempting to speculate that COL is signaled by ITGB1a in gilthead seabream AGs, since it is constitutively expressed by these cells. Nevertheless, this needs further investigation.

The fact that MMP expression levels did not change in AGs following VaDNA and COL1 stimulation but correlated differentially to TIMPs, agrees with our results for *V. anguillarum* infection, pointing to a refined regulation of constitutive MMP activity by TIMP during inflammation. Variations in the correlation between COL1A1 and ITGB1a agree with their role in the signaling modulation of inflammation, innate immune response and wound healing in circulating mammalian monocytes and neutrophils (De Fougerolles and Koteliansky, 2002; Li et al., 1998; Mócsai et al., 2006). In that sense, the stimulation of AGs with COL1 would not result in COL1A1 mRNA induction, unlike VaDNA stimulation, suggesting that COL acts as intermediate signal in the activation of gilthead seabream phagocytes.

In summary, the strongly interactive ECM-related molecule scenarios of inflammation and immunity studied here (DAMP and PAMP stimulations, injury and bacterial infection) underlines the fact that regulation of the ECM environment requires very specialized mechanisms. Moreover, ECM-related molecule roles in inflammatory processes and immune response would be mainly under the control of pro-inflammatory molecules, such as IL-1β, with the particular contribution of COL1 to enhance phagocyte activation. In that sense, ITGB1a appears to be an important constitutive receptor to signal the innate immune response pathways, as in higher vertebrates. Among the ECM-related molecule genes considered, our results also suggest that the *de novo* synthesis of MMPs is modulated mostly in organs with low basal expression, while in organs showing higher constitutive expression levels, such as the head kidney, the activity of MMPs would be regulated by the induction of TIMPs. Of note, too, is the ubiquitous expression of TIMP-2a, which suggests it behaves as a basal regulatory axis of MMP activity (probably equivalent to mammalian TIMP-1), with TIMP-2b playing a more specific role in inflammation and immunity. Finally, our findings suggest that the correlation between ECM-related molecules can be used as a biomarker for early inflammation processes, in accordance to the described usefulness of MMPs correlation with other ECM-related molecules in the assess of endometriosis (Salata et al., 2008) and myocardium damage (Ceauşu et al., 2009). In that respect, its association with other disease related parameters would supplement the individual and ratios data, to achieve more accurate pathophysiologic profiles.

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Competing interests

The authors have declared that no competing interests exist.

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3. A ROLE FOR SPECIFIC COLLAGEN MOTIFS DURING INFLAMMATORY AND WOUND HEALING RESPONSES OF FIBROBLASTS OF THE TELEOST FISH GILTHEAD SEABREAM

A role for specific collagen motifs during inflammatory and wound healing responses of fibroblasts of the teleost fish gilthead seabream

Patricia Castillo-Briceño, Department of Cell Biology and Histology, University of Murcia. Murcia, 30100, Spain.
Dominique Bihan, Department of Biochemistry, University of Cambridge. Cambridge, CB2 1QW, United Kingdom.
Michael Nilges, Department of Structural Biology and Chemistry, Institut Pasteur, 75724 Paris, France.
José Meseguer Peñalver, Department of Cell Biology and Histology, University of Murcia. Murcia, 30100, Spain.
Samir Hamaia, Department of Biochemistry, University of Cambridge. Cambridge, CB2 1QW, United Kingdom.
Alfonsa García-Ayala, Department of Cell Biology and Histology, University of Murcia. Murcia, 30100, Spain.
Richard W. Farndale, Department of Biochemistry, University of Cambridge. Cambridge, CB2 1QW, United Kingdom.
Victoriano Mulero, Department of Cell Biology and Histology, University of Murcia. Murcia, 30100, Spain.

Abstract

Collagen (COL) specific sites and sequences to which cells can attach, either directly or through protein intermediaries, has been identified using Toolkits of triple-helical peptides and specific GXX'GEX" motifs, where their affinities differ sufficiently to control cell adhesion and migration independently of the intracellular regulation. We have previously reported that COL-I was able to prime in vitro the respiratory burst and induce a specific set of immune- and extracellular matrix-related molecules in phagocytes of the teleost fish gilthead seabream (*Sparus aurata* L.). In that sense it was also suggested that COL-I would acts as intermediate signal during the early inflammatory response in gilthead seabream. Since fibroblasts are highly involved in the starting of wound repair and regeneration processes, in the present study we used SAF-1 cells (gilthead seabream fibroblasts) to identify the binding COL motifs by end-point and real-time cell adhesion assays against the Toolkits of COL peptides and motifs. We identified COL motifs involved in the early adhesion of these cells in a magnesium-dependant way. Furthermore, we found that coating GFOGER and GLOGEN motifs (where O is hydroxyproline) present high affinity for SAF-1 adhesion as cell quantity as well as covered surface, while in suspension this motifs were also able to induce the expression of the genes coding for the proinflammatory molecules interleukin-1β and cyclooxygenase-2. These data suggest that specific COL motifs are involved in the regulation of the inflammatory and healing responses of teleost fish.

Key words: adhesion, collagen motifs, fibroblasts, inflammation, *Sparus aurata*, teleost fish.

Introduction

The developmentally complex family of collagens (COLs) is the major component of the extracellular matrix (ECM) of all metazoans [Kramer 1994]. Fibrillar COLs types I and II constitute the most of COL present [Wess 2005], and like the other fibril-forming COLs (types III, V, XI, XXIV and XXVII), they are able to form continuous triple-helical domains [Heino et al. 2009]. These collagens can form stable large fibrils and complex fibrous superstructures that are responsible for the tensile strength of the tissues [Canty and Kadler 2005, Heino et al. 2009, Herr and Farndale 2009].

In addition to their structural role, COLs are proteins able to modulate the cellular inflammatory response and activities depending on the microenvironment and the physiological processes involved. Cellular recognition of COLs is mediated by diverse surface receptors, such as integrins (ITGs), discoidin domain receptors (DDRs), glycoprotein VI (GpVI), etc., which upon COL binding may activate other molecules related to remodelling, inflammation and wound healing processes, such as matrix metalloproteases (MMPs), cytokines and growth factors [Heino 2000, Vogel 2001, Tran et al. 2004, Lee et al. 2007, Leitinger and Hohenester 2007, Heino et al. 2009, Herr and Farndale 2009, Schultz and Wysocki 2009]. COL-I fragments increase the release and activity of interleukin 1 beta (IL-1β) in human peripheral blood- and adherent-monocytes after attachment, such response being mediated by ITG α2β1 (ITGA2B1) and being part of the remodelling mechanisms [Pacifici et al. 1991]. Our findings in the teleost fish gilthead seabream (*Sparus aurata* L.) also showed that native COL-I would act as a damage associated molecular pattern (DAMP) by increasing 1) the respiratory burst in leukocytes, and 2) the mRNA levels of genes coding for IL-1β and other pro-inflammatory molecules in macrophages and acidophilic granulocytes [Castillo-Briceño et al. 2009].

Particular interest on the role of COLs during wound healing and regeneration was given since COL barrier devices are useful to guide these processes, in which COL type, origin and processing may result in differential cellular behaviors [Behring et al. 2008]. These studies have been performed with several cell types, but especially with fibroblasts in accordance to their direct and relevant involvement in different aspects of tissue engineering [Steffensen et al. 2001, Farahani and Kloth 2008, Mazlyzam et al. 2008, Gjorevski and Nelson 2009, Puklin-Faucher and Sheetz 2009, Dobaczewsky et al. 2010]. Fibroblast behaviour modulation by structural ECM molecules like COLs is considered to be triggered by both mechanical and molecular stimulation [Arlein et al. 1998, Steffensen

et al. 2001, Dalby et al. 2007, Barrientos et al. 2008, Daley et al. 2008, Dallon and Ehrlich 2008, Farahani and Kloth 2008, Gjorevski and Nelson 2009, Puklin-Faucher and Sheetz 2009, Jiang et al. 2010, Dobaczewsky et al. 2010]. In example, it is known that in mice fibroblast phenotype and gene expression is altered by their adhesion state [Dhawan et al. 1991], as well as human fibroblasts affinity to a COL surface implicates a modulation in their attachment, morphology, proliferation rate and migration [Rothamel et al. 2004, Behring et al. 2008].

ITGs have been widely studied as mediators of cell binding to COLs and other ECM molecules, especially in the early adhesion mechanisms [Knight et al. 2000, Dobler et al. 2006, Puklin-Faucher and Sheetz 2009]. That binding can be involved in specific cellular processes such as platelets aggregation [Jarvis et al. 2002] or fibroblasts adhesion and migration capacity [Behring et al. 2008]. Some ITG β1 (ITGB1) heterodimers (α1β1, α2β1, α10β1, α11β1) are able to directly bind to COLs by the extracellular I-domain (also called A-domain) of the ITGA subunits [Steffensen et al. 2001, Hynes 2002, Zhang et al. 2003, Raynal et al. 2006, Takada et al. 2007, Bennett et al. 2009], supported by the ITGB1 I-like domain [Xiong and Zhang 2001, Bennett et al. 2009, Isaji et al. 2009, Johnson et al. 2009]. These ITG-COL binding affinity depends on the accessibility to binding sites and the high affinity motifs contained, such as RGDs and GXX'GEX" sequences (where X, X' and X" are variable residues that can be found in this positions) [Knight et al. 2000, Hynes 2002, Jarvis et al. 2002, Zhang et al. 2003, Dobler et al. 2006, Raynal et al. 2006, Leitinger and Hohenester 2007, Takada et al. 2007, Tulla et al. 2007, Al-Jamal and Harrison 2008, Farndale et al. 2008, Heino et al. 2009, Herr and Farndale 2009, Johnson et al. 2009], some of which present a metal-ion (Mg^{2+}, Mn^{2+}, Ca^{2+}) dependent adhesion affinity [Morton et al. 1997, Pesho et al. 2006, Farndale et al. 2008, Heino et al. 2009].

Toolkits of COL-derived peptides and specific motifs are very useful to study the specific sequences involved in receptor recognition of the different types of COLs and the cell response triggered by their binding [Morton et al. 1997, Knight et al. 1998, Verkleij et al. 1998, Knight et al. 2000, Jarvis et al. 2002, Ruehl et al. 2002, Zhang et al. 2003, Dobler et al. 2006, Lebbink et al. 2006, Lisman et al. 2006, Raynal et al. 2006, Farndale et al. 2008, Boilard et al. 2010]. In the present study, we use Toolkits of peptides and specific motifs derived from human COL-II and COL-III, both phylogenetically close to COL-I and contained in the same clade [Heino et al. 2009]. Those Toolkits allow us to elucidate specific COL sequences and motifs that directly induce the early gilthead seabream

fibroblast response in relation to their adhesion affinity and the synthesis of inflammation-related molecules.

Materials and Methods

Cell Culture and Materials

The established cell line of caudal fin fibroblasts from *S. aurata* (SAF-1) were purchased in the ECACC (UK) and cultured at 25 ºC in Leibovitz's L-15 media supplemented with 5 % FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin (Sigma). The COL-II and –III toolkits of triple helical peptides, GXX'GEX" specific motifs, GPP_{10} and CRP ($GPO_{10,}$ where O is hydroxyproline) peptides were synthesized, purified and their triple helical properties tested as previously described [Smethurst et al. 2007]. There were also used the commercial RGD triplet, echistatin (a RGD containing disintegrin), endostatin (carboxyl-terminal domain of collagen XVIII), bovine COL-I (native and denatured at 60 ºC during 0.5 – 3 h), human COL-II, human COL-III and BSA (Sigma), all of them cell culture tested.

End-Point Cell Adhesion Assays

These adhesion assays were performed to evaluate the early attachment affinity of fish fibroblasts with different COL derived peptides, motifs and related sequences. Plastic 96 well plates for ELISA (Immulon 2 and Nunc) were coated by triplicate with 100 µl per well of the COLs, toolkit peptides, GXX'GEX" motifs, GPP_{10} and CRP peptides at 10 µg/ml in 0.01 M acetic acid for at least 16 h at 4 °C based in previous studies [Onley et al. 2000, Smethurst et al. 2007]. While the same process but with different concentrations (0.5 – 50 µg/ml) was performed to test RGD triplet, echistatin and endostatin. Then, plates were blocked with 5 % BSA in TBS (50 mM Tris-HCl, 140 mM NaCl, pH 7.4) during 1 h at 20 ºC and washed to add the cell suspension. Cells were detached following the ECACC specifications, resuspended in 0.1 % BSA in TBS and incubated at 1 x 10^5 cells per well during 50 minutes at 20 ºC, in the presence of 5 mM Mg^{2+} to enhance or 5mM EDTA to inhibit the ITGs extracellular domain binding [Farndale et al. 2008]. Thereafter, plates were washed and adhered cells were incubated 1 h with lysis buffer (0.07 M tri-sodium citrate, 0.3 M citric acid, 0.1 % Triton X-100) to estimate the proportion of attached cells

as the amount of their LHD content using the LDH cytotoxicity kit (Roche) in a FLUOstart luminometer (BGM LabTechnologies).

Real Time Cell Adhesion (RTCA) Assays

RTCA plates (Roche) were coated and blocked similarly to the end-point adhesion assays, but during 1 h each step and at 20 ºC. Next, cells in suspension were added with Mg^{2+}or EDTA, or with Mg^{2+} and 50 µg/ml genomic DNA of *Vibrio anguillarum* (VaDNA) as a pathogen associated molecular pattern (PAMP), and leaved to incubate at 23 ºC. The surface covering was continuously read in a RTCA analyzer (Roche), with measures each 5 min during the first 3 h, then each 20 min until at least 16 h.

Modeling Analysis of the I-like domain of gilthead seabream ITGB1a in complex with COL motifs

We modeled the ITGB1a I-like domain on the basis of its sequence similarity with the extra-cellular extracellular domain of ITGB3 heterodimers (1U8C, ITXV) [Xiao et al. 2004, Xiong et al. 2004], using BLAST (NCBI) to search for homologous domains in the PDB [Berman et al. 2000], T-Coffee [Notredame et al. 2000] to align the sequences and Modeller [Eswar et al. 2006] for the actual modeling. This was performed in an automated way with the BisKit molecular modeling toolkit [Grünberg et al. 2007]. Models of the GFOGER, GLOGEN and CRP motifs were obtained by modifying side-chains in the collagen part of the ITGA2 I-domain in complex with the GFOGER motif (PDB code 1DZI) [Emsley et al. 2000], using ad-hoc scripts developed for CNS [Brunger et al. 1998]. The model of a complex between the ITGB1a I-like domain and the GFOGER, GLOGEN and CRP were obtained by structure superposition of the ITGB1a I-like domain onto the ITGA2 I-domain in the complex with the GFOGER motif. The models were energy minimized and refined in a 12 Å layer of explicit TIP3P solvent model [Jorgensen et al. 1983] with CNS, with the CHARMM Param19 force field [Brooks et al. 1983]. The electrostatic analysis was performed using the ACE generalized Born model implemented in CNS [Calimet et al. 2001]. The difference in energy was estimated as the difference between the electrostatic, van der Waals and generalized Born contributions to the total energy calculated in the complex and the two molecules separated by 100 Å. We used 4 for the internal dielectric and 80 for the external dielectric.

Analysis of Gene Expression

Fish fibroblasts were seeded in TBS supplemented with 5 mM Mg^{2+} at 1 x 10^6 cells in 25 cm^2 flasks which were coated as described for the End-point cell-adhesion assays with COL derived peptides or only BSA. GFOGER and GLOGEN specific motifs were also tested in suspension (10 μg/ml) in BSA coated flasks. Then, cells were incubated during 50 minutes and collected separating the adhered and the non-adhered fractions to be stored in TRIzol at -80 ºC until processing. Samples in TRIzol were thawed on ice and processed for RNA extraction, and then RNA samples were treated with DNase I (1U/μg RNA) Amplification grade (Invitrogen) following the manufacturer's instructions. The RT-qPCR was performed using a SuperScript III ReverseTranscriptase Kit (Invitrogen) to synthesize first strand cDNA from 1 μg of total RNA, and SYBR Green PCR Core Reagents (Applied Biosystems) with an ABI PRISM 7500 instrument (Applied Biosystems) according to the respective manuals and performed by triplicate. For each mRNA, gene expression was normalized to the ribosomal protein S18 content in each sample using the comparative Cq method (ΔΔCq) as previously described [Castillo-Briceño et al. 2009, 2010]. The primers used are shown in Table 3.

Statistical analysis

The data were analyzed by a two-way analysis of variance (ANOVA) and a Bonferroni's post-tests comparing to the respective controls and comparing each type or condition to all the others. All the analyses were performed following the recommendations of the statistical software used (Prism 5 for Windows version 5.00) in accordance to the number of conditions, factors, replicates, repeated measures, distribution, etc. In all the cases significance was considered for $p < 0.05$.

Results

Cell attachment dependent of Mg^{2+} specifically differs among COL-related peptides

End-point adhesion assay results showed that SAF-1 cells have higher affinity for early attachment to the GLOGER-, GFOGER- and GLKGENGLOGEN- containing

peptides in the context of COL-II (Figure 1, Table 1) and COL-III (Figure 2, Table 2) Toolkits. Although other peptides without GXX'GEX" motifs, but with the RGD sequence, also presented high affinity, that is the case of COL-II-51 (Figure 1), COL-III-51 and COL-III-52 peptides (Figure 2), which match the sequence GPQGPRGDK (Table 1, Table 2). The exceptions were the peptides COL-II-21, COL-II-22 (Table 1) and COL-III-9 (Table 2) which do not contain any of the mentioned sequences, but presented a moderate affinity. Further, the assay with specific COL-related motifs and fragments shown in Fig. 3 revealed that 1) the most of the GXX'GEX" motifs were able to support the cell adhesion with the highest affinity for GFOGER, GLKGEN and GLOGEN; 2) only GFOGER and GLOGEN specific motifs reach significant higher affinity than COL-II and –III; 3) RGD triplet showed a moderate affinity whilst echistatin affinity was highly dependent of the concentration, reaching its maximum at 10 μg/ml; 4) native COL-II and COL-III but not COL-I exhibited high affinities, however COL-I denatured form was able to double its affinity, whilst endostatin supported a moderate attachment.

GFOGER and GLOGEN promote a high cell covering of surface *in vitro*

In addition to end-point data, the real-time adhesion assay results (Figure 4) revealed that *in vitro* SAF-1 cells early covering of surface was similar for native COL-I, COL-II and COL-III as for the COL-II-51 peptide compared to GPP10, CRP and BSA controls. It was also not significantly modified with the peptides containing GXX'GER-sequences which presented a low - moderate supporting of surface covering, while COL-III-7 peptide that contain GLKGEN and GLOGEN sequences together (Table 3) supported the highest covering. In the context of specific COL motifs, SAF-1 cells increased the covering of surface only with GFOGER and GLOGEN motifs.

Cell adhesion affinity to COL-derived peptides is strongly modulated after bacterial DNA stimulation

The early cell adhesion affinity for GLOGEN- and GFOGER- containing peptides and specific motifs, measured as the rate of surface covering during the first hour, show a slight increase in VaDNA-stimulated cells. However, this increment was minimal compared to the drastic VaDNA induction of cell adhesion to COL-II-51 peptide and GPP10, CRP and BSA controls, which reached the highest rates of covering (Figure 5).

In the following hours, cell covering surface reach its maximum after 5 – 6 h for all the peptides in the Mg^{2+} supplemented cells, whilst in VaDNA stimulated cells it occurs earlier at 3 – 4 h (excepting COL-II-28 and COL-III-7 peptides at 4 – 5h), doubling the covering reached with Mg^{2+} (Figure 6). After that, cells started detaching with the most of peptides and particularly fast in VaDNA stimulated cells (especially for GLOGEN motif), except for COL-II-28 and COL-III-7 peptides with Mg^{2+}, and only COL-III-7 with VaDNA, which were able to support the cell attachment after at least 16 h. In all the cases, EDTA controls presented a very low cell adhesion rate (Figure 5) and covered surface (Figure 6).

GFOGER and GLOGEN motifs in suspension increase IL-1β mRNA expression in gilthead seabream fibroblasts

The early adhesion of SAF-1 fibroblasts to COL-derived peptides resulted in significant induction of IL-1β expression in adhered cells compared to non-adhered ones, even in the BSA negative control. In coating peptides, there was non-relevant modulation between them, while in suspension it was a clear increase of IL-1β with GFOGER, GLOGEN and COL1 compared to BSA control but just in adhered cells. That differs from VaDNA stimulated cells, which showed a high increase of IL-1β in both adhered and non-adhered cell fractions compared to their correspondent BSA control (Figure 7). In addition, cyclooxygenase 2 (COX-2) presented the same tendency, except for GLOGEN which have non-significant changes even in suspension, while transforming growth factor β1 (TGFB1) expression was non-relevantly modulated in all the cases (data not showed).

The ECM related molecules analyzed (MMP13, COL1A and ITGB1a) only showed a notable significant induction for ITGB1a expression in non-adhered cells compared to the adhered (data not showed). However, in all the immune- and ECM-related genes analyzed we found that the expression level variations were significantly related not only to the peptides but also to their interaction with the adhesion status of the cells (Table 4).

Modeled ITGB1a-GFOGER and -GLOGEN complexes present higher electrostatic stability than ITGB1a-CRP control complex

The modeling analysis of the putative complexes formed between the ITGB1a I-like domain and the GFOGER, GLOGEN or CRP motifs showed that the models with

GFOGER and GLOGEN specific motifs have more favorable electrostatic energies in complex than the CRP control peptide (Table 5), using Mg^{2+} in the active binding site, in agreement with the results from the adhesion assays. This suggests that the sequences may have the same binding mode to the ITGB1a I-like domain studied here as to the ITGA2 I-domain, despite their large sequence difference.

Discussion

Fibroblast adhesion to COL and ECM structural components like fibronectin and laminin has been widely described and considered an important issue of study in relation to morphogenesis, tissue remodeling, wound healing and injury repair processes in mammals, with also a few studies in other vertebrate models. In that sense, over the limitations of cell lines as experimental models, SAF-1 fibroblasts were very useful to assess the COL motifs affinity in a Mg^{2+} dependent manner in fish, as previously performed with constitutively adherent cell lines in mammals [Grenz et al. 1993, Xiong and Zhang 2001, Zhang et al. 2003, Salas et al. 2004, Siljander et al. 2004, Raynal et al. 2006, Farndale et al. 2008]. Further, our results would improve this knowledge in relation to teleost fish fibroblast behavior and allowed us to identify specific COL motifs involved in the early adhesion of gilthead seabream fibroblasts.

During early fish fibroblast attachment, the lower GLOGER and higher GFOGER motif affinities compared to their respective containing peptides COL-II-7 and COL-II-28 suggests that the surrounding sequence is important, as described for GLSGER (COL-III-46) and human platelets [Siljander et al. 2004, Herr and Farndale 2009]. It could also relates to the effect of peptides 3D structure and the helix conformation as previously stated for the GFOGER binding affinity with human fibrosarcoma cells and platelets, and rat Rugli cells [Knight et al. 2000, Heino 2007, Herr and Farndale 2009].These considerations also support the very high affinity of COL-III-7 peptide, where the GLOGEN motif high affinity could be improved by the GLKGEN motif, which offers a moderate-high affinity for attachment. This general considerations would also apply for the other GXX'GEX" motifs analyzed whose attachment affinity was low-moderate, while in the toolkits context it was non-significant comparing to their respective BSA and EDTA controls.

Early attachment affinity levels for GXX'GEX" specific motifs and the fish fibroblast can be then summarized as GLOGEN ≈ GFOGER > GLKGEN > GFPGER ≈

GLOGER ≈ GLOGEA > GMOGER > GAOGER ≈ GPP10 > CRP ≈ BSA based on the significant differences between groups ($p<0.05$). This is in agreement with the stated for GLOGEN and GFOGER as high affinity motifs for cell binding [Raynal et al. 2006, Heino 2007, Farndale et al. 2008, Gigout et al. 2008, Herr and Farndale 2009], while the other motifs could present a low-moderate affinity [Munnix et al. 2008, Herr and Farndale 2009], although the specific affinity levels for each motif vary from the findings in mammalian cells. This difference are unlikely due to phylogenetic reasons since these GXX'GEX" sequences and location are very well conserved in vertebrate COLs [Heino et al. 2009], but it could be due to the cell context and the binding receptors involved [Jarvis et al. 2002, Siljander et al. 2004, Munnix et al. 2008].

Additional to early attachment affinity for GXX'GEX" motifs, surface covering rates could also be summarized as GLOGEN ≈ GFOGER > GFPGER ≈ GLOGER ≈ GMOGER ≈ GAOGER ≈ GROGER ≈ GLKGEN ≈ GLOGEA ≈ GPP10 ≈ COL-III > COL-I ≈ COL-II ≈ CRP ≈ BSA, indicating that GLOGEN and GFOGER are not just able to support early attachment but to induce the cell spreading in fish fibroblasts. The other motifs, their containing peptides (included GFOGER containing peptide) and the native COLs (COL-I, -II and –III) tested, even when the most of them support a significant increase of early attachment, they were not able to modulate the surface covering and by the way the cell spreading. Altogether, the adhesion parameters affinity levels of the COL motifs and SAF-1 cells coincides with the behavior of human platelets [Munnix et al. 2008] and fibrosarcoma cell line HT1080 [Farndale et al. 2008]. This agrees with the general implication of morphology (in constitutively adherent cells) like an indicator of biocompatibility and affinity to a substratum, where flattened cells should be more strongly adhered than cells with a rounded appearance which also would exhibit lower proliferation rates [Rothamel et al. 2004, Behring et al. 2008].

This adhesion affinity profile for GXX'GEX" related peptides leads to suppose that the binding to these motifs by fish fibroblast are mediated by ITGB1 heterodimers containing an extracellular I-domain, particularly because its described high Mg^{2+} dependent affinity for GLOGEN and GFOGER [Perret et al. 2003, Raynal et al. 2006, Heino 2007, Farndale et al. 2008, Herr and Farndale 2009]. Further, it is probably more related to ITGA1B1 and ITGA10B1 binding, considering that GLOGER, GMOGER and GROGER presented a relative affinity notoriously lower than the founded for ITGA2B1 and ITGB11A1 in human and rodents [Raynal et al. 2006, Farndale et al. 2008, Munnix et al. 2008].

About COL peptides containing the RGD sequence, it appears like this triplet is the affinity motif for their early attachment with fish fibroblast as the RGD good binding affinity described across animal species [Morton et al. 1997, Dobler et al. 2006, Heino 2007, Gigout et al. 2008, Heino et al. 2009] and mainly mediated by the I-like domain of ITG beta subunits [Raynal et al. 2006], considering that all the RGD containing peptides showed very similar levels of early cell attachment, almost independently of the location in the strand and the surrounding residues, which can be discarded from the overlapping sequences, and because the non-COL and non-triple helix peptide echistatin reach the same affinity levels at the same concentration of COL peptides (10 μg/ml). However, since RGD triplet alone reach only low-moderate levels it would need a peptide context to improve their affinity as suggested before in relation to fibronectin [Takagi 2004, Daley et al. 2008]. This attachment would be corresponding with the RGD-ITG binding participation during early cell signaling by ECM molecules [Barczyk et al. 2010], but would not be involved in cell spreading and firm adhesion as suggested by the inability of the RGD containing peptide COL-II-51 (different to the high affinity GXX'GEX" motifs) to induce the fish fibroblasts spreading. This is again in agreement with the kinetics results for ITGA5B1-RGD (in fibronectin) that suggests RGD as a facilitator of the initial encounter rather than a contributor to more stable protein–protein interactions of the cell surface with the ECM structural components [Takagi 2004]. In addition, COL-II-21 and COL-II-22 early attachment affinity could also be related to RGD as these peptides content a DGR sequence which is described as able to support a cell binding (although in levels lower than RGD) with endothelial cells and mediated by ITGAVB3, where DGR would perform as a precursor of isoDGR and act as a regulator of the protein functions being recognized similarly to RGD [Curnis et al. 2006]. This could also be the case of COL-III-9 peptide that after the analysis of its sequence in the Toolkits context showed GARGND sequence as a candidate, as it is present just in this peptide and in COL-II-8 where would be improving the affinity to GLOGER motif (which is moderate in SAF-1), probably by the RGN triplet as a precursor of a more active form related to RGD binding to ITGB I-like domains as described for its inversed form NGR [Takahashi et al. 2007, Spitaleri et al. 2008, Xu et al. 2010]. Similar ITGB binding mechanism is also proposed for endostatin as its cell interactions are suggested to be mediated by an RGD-dependent integrin [Rehn et al. 2001].

It was also remarkable that in the toolkits context COL-II contained more adhesion affinity sites than COL-III for fish fibroblasts, although both of them in the native for

showed similar affinity. This probably is due to a cryptic location of the binding affinity sites in the native COLs which would be recognized by the cell receptors only when exposed [Zhang et al. 2003, Heino 2007, Heino et al. 2009] in denatured forms or like in this case in synthetic short peptides. It is also supported by the COL-I affinity increasing after its denaturing.

Considering the high complex stability predicted for gilthead seabream models of I-like domain of ITGB1a with GFOGER and GLOGEN compared to CRP, suggest a binding affinity of this receptor subunit similar to the demonstrated for ITGA2, which binding to GFOGER motif is mediated by its I-domain in a Mg^{2+} dependent manner [Emsley et al. 2000]. In consequence, it is tempting to speculate that the ITGB1b I-like domain would be acting as an extracellular receptor directly involved in GXX'GEX" motifs binding by fish fibroblasts and it would not be totally depending on the I-domain of the ITGA subunit, more as this domain is at moment only predicted in fish while ITGB1a was constitutively expressed in all the tissues of gilthead seabream [Castillo-Briceño et al. 2010]. If that is really occurring, this binding mechanism would correspond with ITGB1 I-like domain ligation to ECM proteins when there is lack of ITGA I-domain in the ITG heterodimer [Takagi and Springer 2002, Barczyk et al. 2010]. However, it is clear that directly or indirectly, ITGB1a have an important participation during fish fibroblast initial adhesion, supported by its upper expression in suspended fibroblasts compared to the adhered ones and because COL motifs of high affinity for these cells correspond to the target binding sequences for ITGB1 heterodimers in other vertebrates as stated before for GXX'GEX" and RGD related peptides [Raynal et al. 2006, Farndale et al. 2008, Munnix et al. 2008, Heino et al. 2009].

Interestingly, PAMP (VaDNA) stimulation can result in a dramatic generalized induction of surface covering by fish fibroblasts during the early attachment, where the relative affinity levels for the adhesion substrates is inversed to the following GPP10 ≈ COL-II-51 (RGD) ≈ BSA ≈ CRP ≥ COL-II-28 (GFOGER) ≈ COL-III-7 (GLOGEN) > GFOGER ≈ GLOGEN. It is easy to suppose that this behavior could constitute a mechanism of primary defense related to wound healing, where the PAMPs activated fibroblasts prompt to a rapid formation of a layer to perform as a barrier against exogenous agents, in agreement with the relation between the activation state of the cell and its behavior related to adhesion to GXX'GEX" motifs [Farndale et al. 2008]. Thus, fibroblast responses to PAMPs would be also regulated by the substrate, as demonstrated by the low relative surface covering with the GXX'GEX" high affinity peptides, more significant on

the part of GFOGER and GLOGEN specific motifs. This role for the GXX'GEX" agrees with the highly studied capacity of COLs and their derived peptides to differentially guide the different phases of wound healing or tissue repairing and regeneration processes, such as fibroblasts adhesion and hemostasis [Ruehl et al. 2002, Rothamel et al. 2004, Leitinger and Hohenester 2007, Wolf et al. 2009, Damodarasamy et al. 2010].

However, this modulation would not depend only on the cell binding to specific motifs but also to the substrate mechanical properties and the forces related to the cell-substrate interactions that modulate the biological activity of cells (such as spreading, contraction, migration and division), altering the micro- and macro-environmental dynamics [Farahani and Kloth 2008, Damodarasamy et al. 2010, Jiang et al. 2010]. This could partially explain the later detachment of fish fibroblast after reaching the maximal cell adhesion, especially notorious for GLOGEN related peptides. GLOGEN as specific motif is able to support a Mg^{2+} dependent cell adhesion during the early attachment (< 1 h), then cell spreading should be induced and progressively increased to reach their maximum in the middle term (4-6 h), holding this adhesion until a cell detachment start. This behavior is enhanced and accelerated in the presence of a PAMP, but this effect would be also regulated by the residues surrounding GLOGEN in the context of the COL fibers where the attachment is supported for significant longer periods (> 16 h). It converges with the findings in human fibroblasts seeded in COL matrices, whose initial adhesive interactions (1 h) appeared mediated by ITGB1 receptors in a punctuate distribution, after fibroblasts encounter resistance in the matrices their morphology changes to stellate/bipolar and the cell–matrix interactions mature to a focal adhesion organization (4 h), which is stronger than the punctuate but dependent of the presence of growth factors, to be followed by a matrix remodeling [Tamariz and Grinnell 2002].

These fibroblast mechanisms occurred during adhesion match with the described for primary and secondary wound closure, where attachment, proliferation and migration of fibroblasts on a collagen membrane are a prerequisite. In this scenario, cells adherence to COL surface after only 1 h was found to need longer time to finish the attachment process since at 1.5 h attached cells still rounded in shape, while after 5 days fibroblasts had a spindle-shaped to flat morphology on all collagen membranes tested [Behring et al. 2008]. Wound healing processes include specific highly integrated and overlapping phases such as hemostasis, inflammation, proliferation, formation of granulation tissue, reepithelialization, matrix formation, tissue remodeling or resolution [Barrientos et al. 2008, Guo and DiPietro 2010], in which the relevant role of ECM components is widely

known [Tran et al. 2004]. In that context, it is possible to speculate that SAF-1 cells in this model mimic the general behavior of fibroblasts in response to wound healing, where the significant increased expression of IL-1β and COX-2 genes (but not of TGFB1, MMP-13 and COL1A1 genes) after adhesion and during the early attachment should be part of the inflammatory phase. Interestingly, the higher increase of pro-inflammatory molecules when the COL peptides where present in suspension instead of immobilized as substrate corresponds with the described role for COL as a damage signal in fish [Castillo-Briceño et al. 2009] and its suggested activity as intermediate signal in the activation of fish phagocytes [Castillo-Briceño et al. 2010]. That would relate to the mechanisms of signaling for the called "defense COLs" and their role in the development of an appropriate immune response and the clearance of pathogens from the organism [Fraser and Tenner 2008]. This effect, at least in the case of fish fibroblasts, seems to be non-dependent of the specific GXX'GEX" motifs or the cell adhesion affinity, since GLOGEN and GFOGER motifs effects were not significantly different to the levels with native COL-I.

In addition, cell detachment after the maximal binding can relates to the resistance to fibroblasts contraction that is widely described for 3D COL matrices and scaffolds, and related to wound healing [Steffensen et al. 2001, Dallon and Ehrlich 2008, Van der Veer et al. 2009, Corin and Gibson. 2010, Guo and DiPietro 2010], and which is enhanced depending of several facts like low strength of the COL substrate [Daley et al. 2008, Farahani and Kloth 2008, Damodarasamy et al. 2010], presence of TGFBs [Ferguson and O'Kane 2004, Daley et al. 2008, Dallon and Ehrlich 2008, Farahani and Kloth 2008,] and ITGs binding to COL [Steffensen et al. 2001, Dallon and Ehrlich 2008, Gjorevski and Nelson 2009, Van der Veer et al. 2009]. In this sense, the posterior maintenance of fish fibroblasts attachment to GLOGEN and GFOGER containing peptides would depends of the mechanical properties due to the surrounding residues (and its interactions with the cell surface and the support where they are immobilized), and the availability of modulator molecules produced by the cells, like TGFB1, which was not significantly modulated but constitutively expressed in all the cases.

Finally, we could conclude that SAF-1 cells are a feasible model to study the adhesive affinity and recognition of COL peptides in fish, exhibiting a Mg^{2+} affinity profile of adhesion very similar to the described for mammalian I- and I-like domain in ITGB1 heterodimers. Moreover, GXX'GEX" motifs are able to differentially modifies their behavior and pro-inflammatory genes expression profile, being useful for posterior studies

in wound healing mechanisms and involvement of COL during the early inflammatory response.

Authors' contributions

PC-B, AG-A, RWF and VM were responsible for conceiving, designing and coordinating the study. PC-B made substantial contributions to the data acquisition (cell culture, adhesion assays, mRNA extraction and processing for qRT-PCR, searching of data bases for the bioinformatics' processing), analysis and interpretation, and wrote the manuscript. DB synthesized the COL derived peptides and GXX'GEX" motifs used in the studio. MN realizes the bioinformatics' analysis of the ITG-COL peptides complex stability and contributes with the revision of the methodology and discussion. SH provide initial advice and assistance on adhesion assays. JM contributed to the revision and edition of the manuscript. AG-A, RWF and VM made substantial contributions to the interpretation of data, and the manuscript revision and edition, they contributed equally to this work. All authors read and approved the final manuscript.

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Competing Interests

The authors have declared that no competing interests exist.

Abbreviations

Cq, quantification cycle; VaDNA, genomic DNA from *Vibrio anguillarum*.

Figure Legends

Figure 1

Gilthead seabream fibroblasts (SAF-1) present differential early attachment affinity to COL-II toolkit peptides. Cells were incubated during 50 minutes in the presence of 5 mM Mg^{2+} to enhance or 5 mM EDTA to inhibit the I-domain and I-like domain mediated cell binding. GXX'GEX" containing peptides (II-7, -8, -28) and RGD related peptides (II-8, 21, -22, -32, -41, -51) showed significantly higher adhesion affinity compared to their EDTA and BSA controls (* is according to Bonferroni test from a 2-way ANOVA, $p < 0.05$). Each bar represents the mean ±S.D. of two independent experiments performed by triplicate.

Figure 2

Gilthead seabream fibroblasts (SAF-1) present differential early attachment affinity to COL-III toolkit peptides. Cells were incubated during 50 minutes in the presence of 5 mM Mg^{2+} to enhance or 5 mM EDTA to inhibit the I-domain and I-like domain mediated cell binding. GXX'GEN containing peptide (III-7) and RGD related peptides (II-9, -51, -52) showed significantly higher adhesion affinity compared to their EDTA and BSA controls (* is according to Bonferroni test from a 2-way ANOVA, $p < 0.05$). Each bar represents the mean ±S.D. of two independent experiments performed by triplicate.

Figure 3

Gilthead seabream fibroblasts (SAF-1) present differential early attachment affinity to specific COL and RGD-related peptides. Cells were incubated during 50 minutes in the presence of 5 mM Mg^{2+} to enhance or 5 mM EDTA to inhibit the I-domain and I-like domain mediated cell binding. Native and denatured (0.5 – 3 h at 60 ºC) COLs, and the COL derived peptides were used at 10 μg/ml. RGD triplet, echistatin (Ech) and endostatin (End) were tested at different concentrations (0.5 – 50 μg/ml as indicated). Native COL-II and -III, and denatured COL-I, as well as the GXX'GEX" specific motifs tested (excepting GAOGER), RGD, Ech and End showed significantly higher adhesion affinity compared to their EDTA and BSA controls (* is according to Bonferroni test from a 2-way ANOVA, $p < 0.05$). Each bar represents the mean ±S.D. of three independent experiments performed by triplicate.

Figure 4

Gilthead seabream fibroblasts (SAF-1) present different surface covering rates affinity to GLOGEN and GFOGER specific motifs. Incubations were in the presence of 5 mM Mg^{2+} to enhance or 5 mM EDTA to inhibit the I-domain and I-like domain mediated cell binding. Surface covering was continuously read and the slope for the first hour calculated. GLOGEN, GFOGER and COL-III-7 peptide were the only with significantly higher spreading affinity compared to their EDTA and BSA controls (* is according to Bonferroni test from a 2-way ANOVA, $p < 0.05$). Each bar represents the mean ±S.D. of three independent experiments performed by triplicate.

Figure 5

Pathogen associated molecular pattern stimulation drastically modulates gilthead seabream fibroblasts (SAF-1) adhesion affinity. Incubations were in the presence of 5 mM Mg^{2+} to enhance or 5 mM EDTA to inhibit the I-domain and I-like domain mediated cell binding, or 5 mM Mg^{2+} and 50 μg/ml *Vibrio anguillarum* genomic DNA (VaDNA) as a PAMP. Surface covering was continuously read and the slope for the first hour calculated. Mg^{2+} dependent affinity to GXX'GEX" related peptides was slightly increased with VaDNA, but with final levels lower than the reached by the low spreading affinity substrates (COL-II-51, GPP_{10}, CRP and BSA). Letters denote significant different groups, where "a" is equal to EDTA control group (according to Bonferroni test from a 2-way ANOVA, $p < 0.05$). Each dot represents the mean ±S.D. of triplicate samples.

Figure 6

Gilthead seabream fibroblasts (SAF-1) present higher surface covering affinity for GFOGER and GLOGEN motifs in the context of toolkits. Incubations were in the presence of 5 mM Mg^{2+} to enhance or 5 mM EDTA to inhibit the I-domain and I-like domain mediated cell binding, or 5 mM Mg^{2+} and 50 μg/ml *Vibrio anguillarum* genomic DNA (VaDNA). Surface covering was continuously read during at least 16 h. After the maximum covering is reached, cells start detaching except by the GLOGEN and GFOGER containing peptides. This process is accelerated and enhanced in the presence of VaDNA, where only the GLOGEN containing peptide (COL-III-7) was able to support the cell spreading after 16 h. Each curve represents the mean ±S.D. of triplicate samples.

Figure 7

Interleukin-1 beta (*il1b*) gene expression is increased in adhered gilthead seabream fibroblasts stimulated with GFOGER and GLOGEN motifs in suspension. The *il1b* mRNA levels were determined by RT-qPCR in amplification products obtained from SAF-1 cells. Gene expression is normalized against *rps18* and relative to BSA non-adhered cells. Each bar represents the mean ±S.D. of triplicates. Letters denote significant different groups, where "a" or "A" are not significantly different to the respective non-adhered or adhered BSA controls (according to Bonferroni's post-test for a 2-way ANOVA, $p<0.05$).

Tables

Table 1

Sequences of Collagen type II Toolkit Peptides

Peptide	Specific Sequence
1	GPMGPMGPRGPOGPAGAOGPQGFQGNO
2	GPQGFQGNOGEOGEOGVSGPMGPRGPO
3	GPMGPRGPOGPOGKOGDDGEAGKOGKA
4	GEAGKOGKAGERGPOGPQGARGFOGTO
5	GARGFOGTOGLOGVKGHRGYOGLDGAK
6	GYOGLDGAKGEAGAOGVKGES**GSOGEN**
7	GES**GSOGEN**GSOGPMGPR**GLOGER**GRT
8	**GLOGER**GRTGPAGAAGA***RGN***DGQOGPA
9	GNDGQOGPAGPOGPVGPAGGOGFOGAO
10	GGOGFOGAOGAKGEAGPTGARGPEGAQ
11	GARGPEGAQGPRGEOGTOGSOGPAGAS
12	GSOGPAGASGNOGTDGIOGAKGSAGAO
13	GAKGSAGAOGIAGAOGFOGPRGPOGPQ
14	GPRGPOGPQGATGPLGPKGQTGEOGIA
15	GQTGEOGIAGFKGEQGPKGEOGPAGPQ
16	GEOGPAGPQGAOGPAGEEGKRGARGEO
17	GKRGARGEOGGVGPIGPOGERGAOGNR
18	GERGAOGNRGFOGQDGLAGPK**GAOGER**
19	GPK**GAOGER**GPSGLAGPKGANGDOGRO
20	GANGDOGROGEOGLOGARGLTGROGDA
21	GLTGROGDAGPQGKVGPSGAOGE***DGR***O
22	GAOGE***DGR***OGPOGPQGARGQOGVMGFO
23	GQOGVMGFOGPKGANGEOGKAGEKGLO
24	GKAGEKGLOGAOGLRGLOGKDGETGAA
25	GKDGETGAAGPOGPAGPAGERGEQGAO
26	GERGEQGAOGPSGFQGLOGPOGPOGEG
27	GPOGPOGEGGKOGDQGVOGEAGAOGLV
28	GEAGAOGLVGPRGER**GFOGER**GSOGAQ
29	GERGSOGAQGLQGPRGLOGTOGTDGPK
30	GTOGTDGPKGASGPAGPOGAQGPOGLQ
31	GAQGPOGLQ**GMOGER**GAAGIAGPKGDR
32	GIAGPKGD***RGD***VGEKGPEGAOGKDGGR
33	GAOGKDGGRGLTGPIGPOGPAGANGEK
34	GPAGANGEKGEVGPOGPAGSAGARGAO
35	GSAGAR**GAOGER**GETGPOGPAGFAGPO
36	GPAGFAGPOGADGQOGAKGEQGEAGQK
37	GEQGEAGQKGDAGAOGPQGPSGAOGPQ
38	GPSGAOGPQGPTGVTGPKGARGAQGPO
39	GARGAQGPOGATGFOGAAGRVGPOGSN
40	GRVGPOGSNGNOGPOGPOGPSGKDGPK
41	GPSGKDGPKGA***RGD***SGPOGRAGEOGLQ
42	GRAGEOGLQGPAGPOGEKGEOGDDGPS

43	GEOGDDGPSGAEGPOGPQGLAGQRGIV
44	GLAGQRGIVGLOGQRGERGFOGLOGPS
45	GFOGLOGPSGEOGKQGAOGASGDRGPO
46	GASGDRGPOGPVGPOGLTGPAGEOGRE
47	GPAGEOGREGSOGADGPOGRDGAAGVK
48	GRDGAAGVKGDRGETGAVGAOGAOGPO
49	GAOGAOGPOGSOGPAGPTGKQGDRGEA
50	GKQGDRGEAGAQGPMGPSGPAGARGIQ
51	GPAGARGIQGPQGP***RGD***KGEAGEOGER
52	GEAGEOGERGLKGHRGFTGLQGLOGPO
53	GLQGLOGPOGPSGDQGASGPAGPSGPR
54	GPAGPSGPRGPOGPVGPSGKDGANGIO
55	GKDGANGIOGPIGPOGPRGRSGETGPA
56	GPRGRSGETGPAGPOGNOGPOGPOGPO

Candidate sequences for SAF-1 adhesion affinity were highlighted: **GXX'GEX"** motifs, ***RGD*** related peptides, Sequences from overlapping in significant affinity peptides, **other non-GXX'GEX"** possible candidates.

Table 2
Sequences of Collagen type III Toolkit Peptides

Peptide	Specific Sequence
1	GLAGYOGPAGPOGPOGPOGTSGHOGSO
2	GTSGHOGSOGSOGYQGPOGEOGQAGPS
3	GEOGQAGPSGPOGPOGAIGPSGPAGKD
4	GPSGPAGKDGESGRO**GROGER**GLOGPO
5	GERGLOGPOGIKGPAGIOGFOGMKGHR
6	GFOGMKGHRGF***DGR***NGEKGETGAOGLK
7	GETGAO**GLKGENGLOGEN**GAOGPMGPR
8	GAOGPMGPR**GAOGER**GROGLOGAAGAR
9	GLOGAAGA*RGND*GARGSDGQOGPOGPO
10	GQOGPOGPOGTAGFOGSOGAKGEVGPA
11	GAKGEVGPAGSOGSNGAOGQRGEOGPQ
12	GQRGEOGPQGHAGAQGPOGPOGINGSO
13	GPOGINGSOGGKGEMGPAGIOGAOGLM
14	GIOGAOGLMGARGPOGPAGANGAOGLR
15	GANGAOGLRGGAGEOGKNGAKGEOGPR
16	GAKGEOGPRGERGEAGIOGVOGAKGED
17	GVOGAKGEDGKDGSOGEOGANGLOGAA
18	GANGLOGAAGERGAOGFRGPAGPNGIO
19	GPAGPNGIOGEKGPAGERGAOGPAGPR
20	GAOGPAGPRGAAGEOGRDGVOGGOGMR
21	GVOGGOGMRGMOGSOGGOGSDGKOGPO
22	GSDGKOGPOGSQGESGROGPOGPSGPR
23	GPOGPSGPRGQOGVMGFOGPKGNDGAO
24	GPKGNDGAOGKNGERGGOGGOGPQGPO
25	GGOGPQGPOGKNGETGPQGPOGPTGPG
26	GPOGPTGPGGDKGDTGPOGPQGLQGLO
27	GPQGLQGLOGTGGPOGENGKOGEOGPK
28	GKOGEOGPKGDAGAOGAOGGKGDAGAO
29	GGKGDA**GAOGER**GPOGLAGAOGLRGGA
30	GAOGLRGGAGPOGPEGGKGAAGPOGPO
31	GAAGPOGPOGAAGTOGLQ**GMOGER**GGL
32	**GMOGER**GGLGSOGPKGDKGEOGGOGAD
33	GEOGGOGADGVOGKDGPRGPTGPIGPO
34	GPTGPIGPOGPAGQOGDKGEGGAOGLO
35	GEGGAOGLOGIAGPRGSOGERGETGPO
36	GERGETGPOGPAGFOGAOGQNGEOGGK
37	GQNGEOGGKGERGAOGEKGEGGPOGVA
38	GEGGPOGVAGPOGGSGPAGPOGPQGVK
39	GPOGPQGVKGERGSOGGOGAAGFOGAR
40	GAAGFOGARGLOGPOGSNGNOGPOGPS
41	GNOGPOGPSGSOGKDGPOGPAGNTGAO
42	GPAGNTGAOGSOGVSGPKGDAGQOGEK
43	GDAGQOGEKGSOGAQGPOGAOGPLGIA
44	GAOGPLGIAGITGARGLAGPOGMOGPR
45	GPOGMOGPRGSOGPQGVKGESGKOGAN
46	GESGKOGAN**GLSGER**GPOGPQGLOGLA

47	GPQGLOGLAGTAGEOGRDGNOGSDGLO
48	GNOGSDGLOGRDGSOGGKGDRGENGSO
49	GDRGENGSOGAOGAOGHOGPOGPVGPA
50	GPOGPVGPAGKSGDRGESGPAGPAGAO
51	GPAGPAGAOGPAGSRGAOGPQGP***RGD***K
52	GPQGP***RGD***KGETGERGAAGIKGHRGFO
53	GIKGHRGFOGNOGAOGSOGPAGQQGAI
54	GPAGQQGAIGSOGPAGPRGPVGPSGPO
55	GPVGPSGPOGKDGTSGHOGPIGPOGPR
56	GPIGPOGPRGNRGERGSEGSOGHOGQO
57	GERGSEGSOGHOGQOGPOGPOGAOGPC

Candidate sequences for SAF-1 adhesion affinity were highlighted: **GXX'GEX"** motifs, ***RGD*** related peptides, Sequences from overlapping in significant affinity peptides, **other non-GXX'GEX"** possible candidates.

Table 3
Primer sequences used for gene expression analysis. The gene symbols followed the Zebrafish Nomenclature Guidelines (http://zfin.org/zf info/nomen.html).

Gene	**Accession number**	**Primer name**	**Sequence (5'→3')**
rps18	AM490061	F	AGGGTGTTGGCAGACGTTAC
		R	CTTCTGCCTGTTGAGGAACC
il1b	AJ277166	F3	ATGCCCGAGGGGCTGGGC
		R2	CAGTTGCTGAAGGGAACAGAC
cox2	AM296029	F1	GAGTACTGGAAGCCGAGCAC
		R1	GATATCACTGCCGCCTGAGT
tgfb1	AF424703	F	AGAGACGGGCAGTAAAGAA
		R	GCCTGAGGAGACTCTGTTGG
mmp13	AM905935	F	CGGTGATTCCTACCCATTTG
		R	TGAGCGGAAAGTGAAGGTCT
col1a1	DQ324363	F2	GCTCTCAGCCAGAGGATGTC
		R2	TGTAGGCGATGCTGTTCTTG
itgb1a	FN649420	F2	AAGGGAGACGAGTTCAATCGGT
		R1	CACCAGCAGACGAGTCACAT

Table 4
Percentage of gene expression variation related to the COL-derived peptides, cell adhesion and their interactions in SAF-1 cells after 1 h of incubation.

Gene	**% of total gene expression variation**			**P value summary (p<0.05)***		
	Peptide	**Adhesion**	**Interaction**	**Peptide**	**Adhesion**	**Interaction**
il1b	39.70	27.97	21.16	***	***	***
cox2	61.20	4.85	33.39	***	***	***
tgfb1	42.31	0.02	56.69	***	ns	***
mmp13	28.34	25.31	40.83	***	***	***
col1a	62.31	1.72	31.58	***	***	***
itgb1a	32.04	20.27	46.26	***	***	***

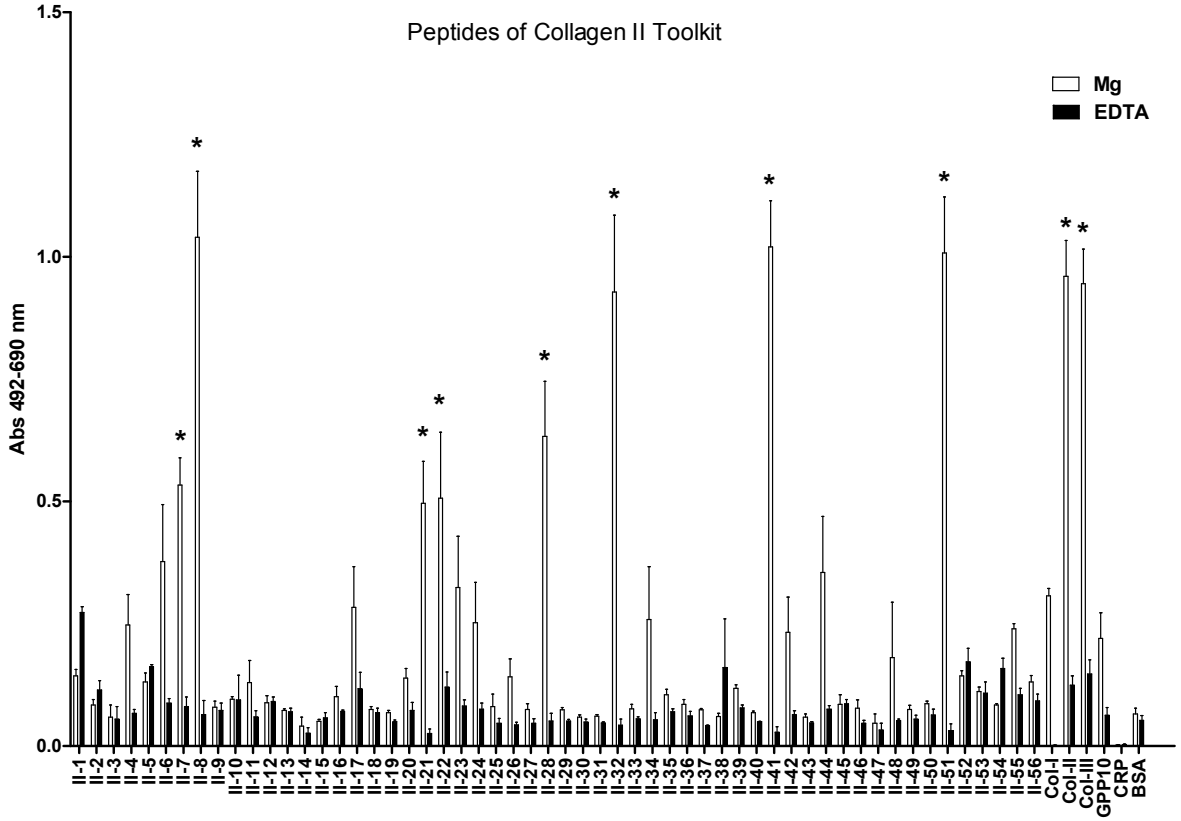
*Significant differences were calculated with a two-way ANOVA and Bonferroni pots-test.
ns= not significant
*** The effect is considered extremely significant

Table 5
Electrostatic stability for ITGB1a and COL specific motifs modeled complexes (free energy of binding)

Complex	ΔG (energy of complex - energy of separated proteins)
ITGB1a-GFOGER	-86 +/- 53 kcal/mol [b]
ITGB1a-GLOGEN	-67 +/- 45 kcal/mol [b]
ITGB1a-CRP	-36 +/- 37 kcal/mol [a]

[a] Letters denotes the groups after a 1-way ANOVA and Bonferroni post-test ($p<0.05$).

Figure 1


Peptides of Collagen II Toolkit
Mg
EDTA
Abs 492-690 nm
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1.0
0.5
0.0
II-1
II-2
II-3
II-4
II-5
II-6
II-7
II-8
II-9
II-10
II-11
II-12
II-13
II-14
II-15
II-16
II-17
II-18
II-19
II-20
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II-25
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II-27
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II-30
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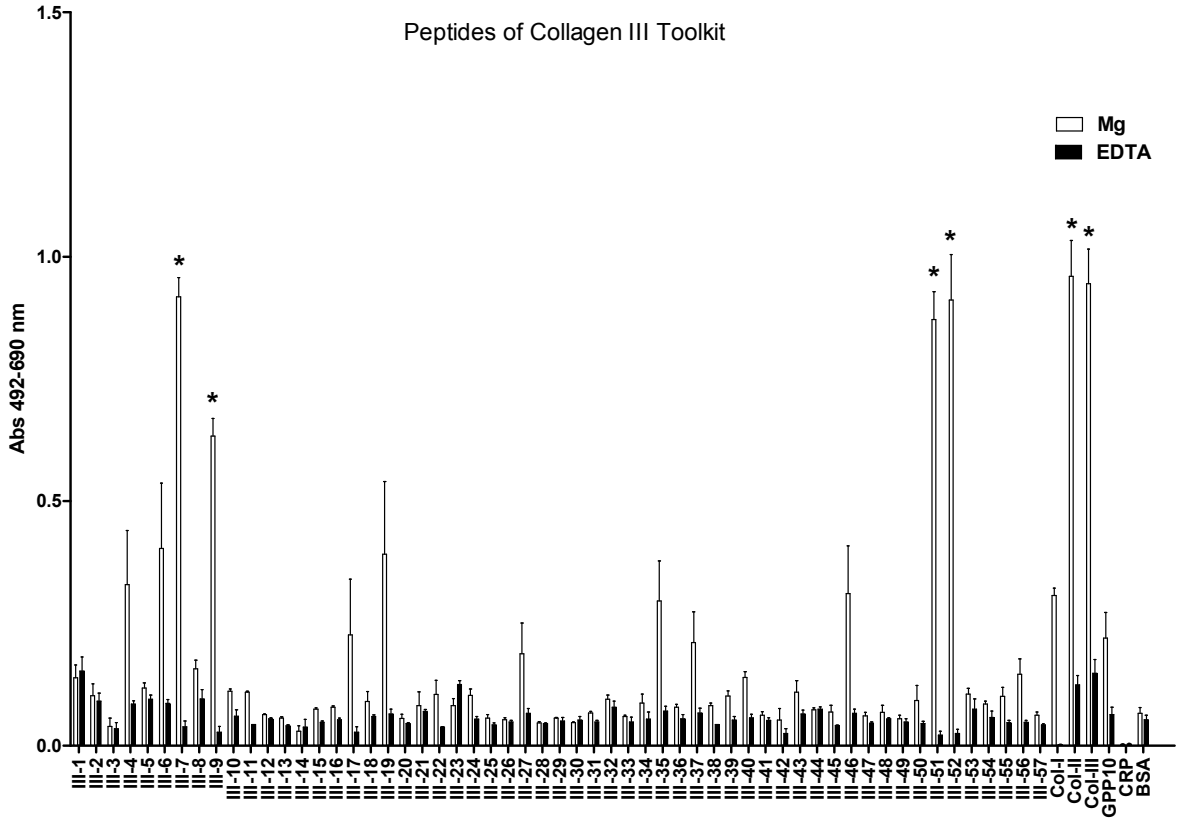
II-32
II-33
II-34
II-35
II-36
II-37
II-38
II-39
II-40
II-41
II-42
II-43
II-44
II-45
II-46
II-47
II-48
II-49
II-50
II-51
II-52
II-53
II-54
II-55
II-56
Col-I
Col-II
Col-III
GPP10
CRP
BSA

Figure 2


Peptides of Collagen III Toolkit
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EDTA
Abs 492-690 nm
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1.0
0.5
0.0
III-1
III-2
III-3
III-4
III-5
III-6
III-7
III-8
III-9
III-10
III-11
III-12
III-13
III-14
III-15
III-16
III-17
III-18
III-19
III-20
III-21
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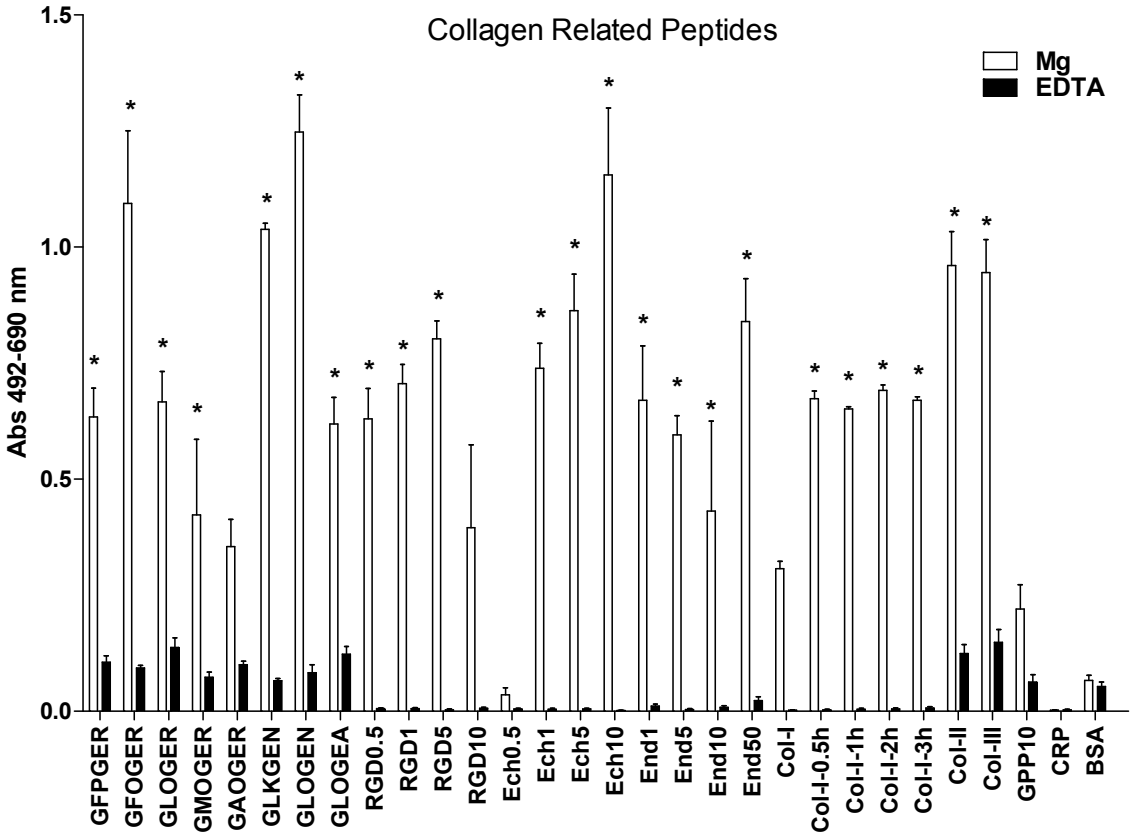
III-34
III-35
III-36
III-37
III-38
III-39
III-40
III-41
III-42
III-43
III-44
III-45
III-46
III-47
III-48
III-49
III-50
III-51
III-52
III-53
III-54
III-55
III-56
III-57
Col-I
Col-II
Col-III
GPP10
CRP
BSA

Figure 3


Collagen Related Peptides
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EDTA
Abs 492-690 nm
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1.0
0.5
0.0
GFPGER
GFOGER
GLOGER
GMOGER
GAOGER
GLKGEN
GLOGEN
GLOGEA
RGD0.5
RGD1
RGD5
RGD10
Ech0.5
Ech1
Ech5
Ech10
End1
End5
End10
End50
Col-I
Col-I-0.5h
Col-I-1h
Col-I-2h
Col-I-3h
Col-II
Col-III
GPP10
CRP
BSA

Figure 4

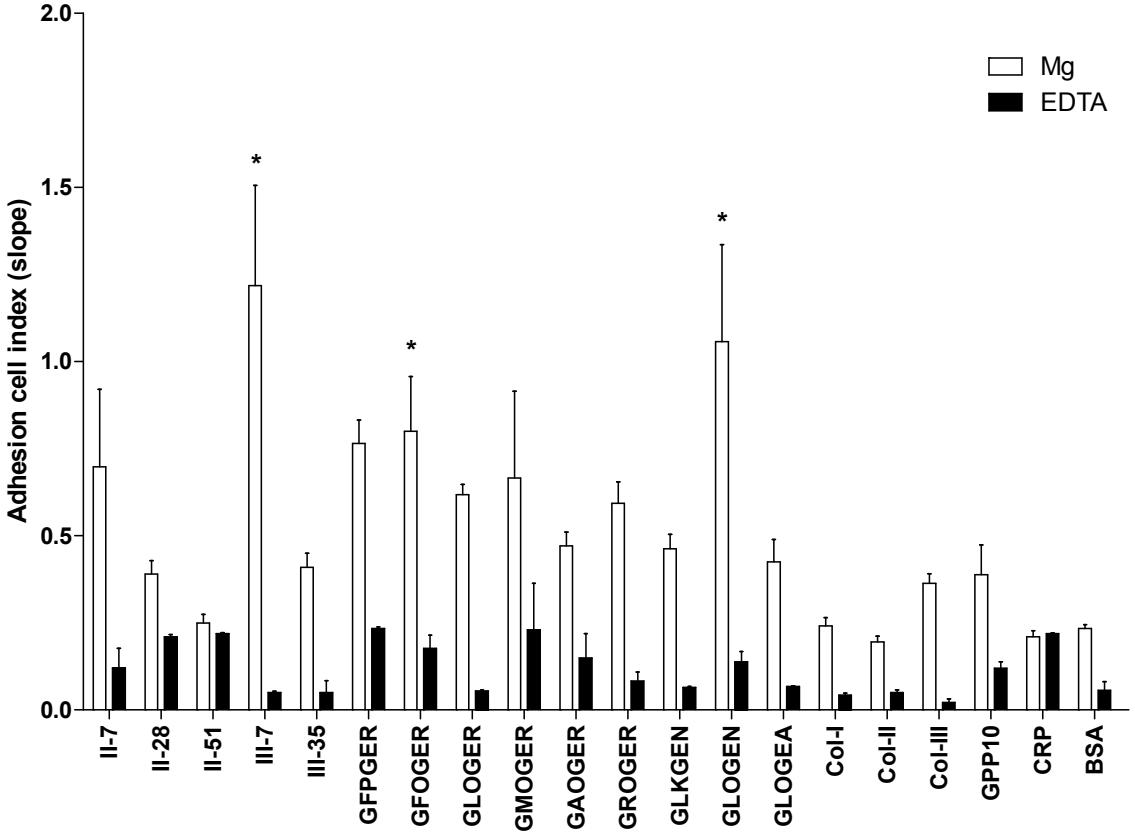

Mg
EDTA
Adhesion cell index (slope)
2.0
1.5
1.0
0.5
0.0
*
*
*
II-7
II-28
II-51
III-7
III-35
GFPGER
GFOGER
GLOGER
GMOGER
GAOGER
GROGER
GLKGEN
GLOGEN
GLOGEA
Col-I
Col-II
Col-III
GPP10
CRP
BSA

Figure 5

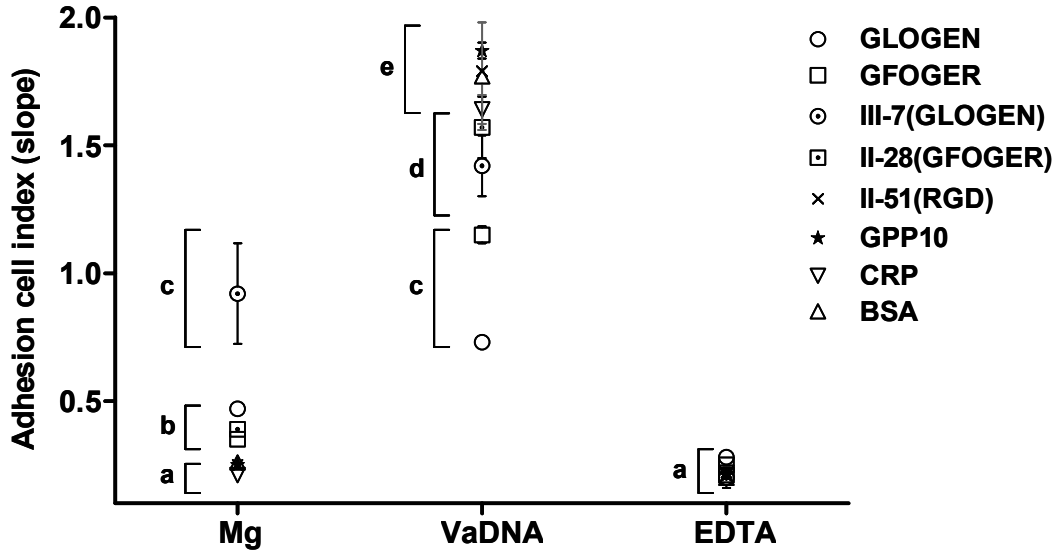

Adhesion cell index (slope)
2.0
1.5
1.0
0.5
a
b
c
c
d
e
a
Mg
VaDNA
EDTA
GLOGEN
GFOGER
III-7(GLOGEN)
II-28(GFOGER)
II-51(RGD)
GPP10
CRP
BSA

Figure 6

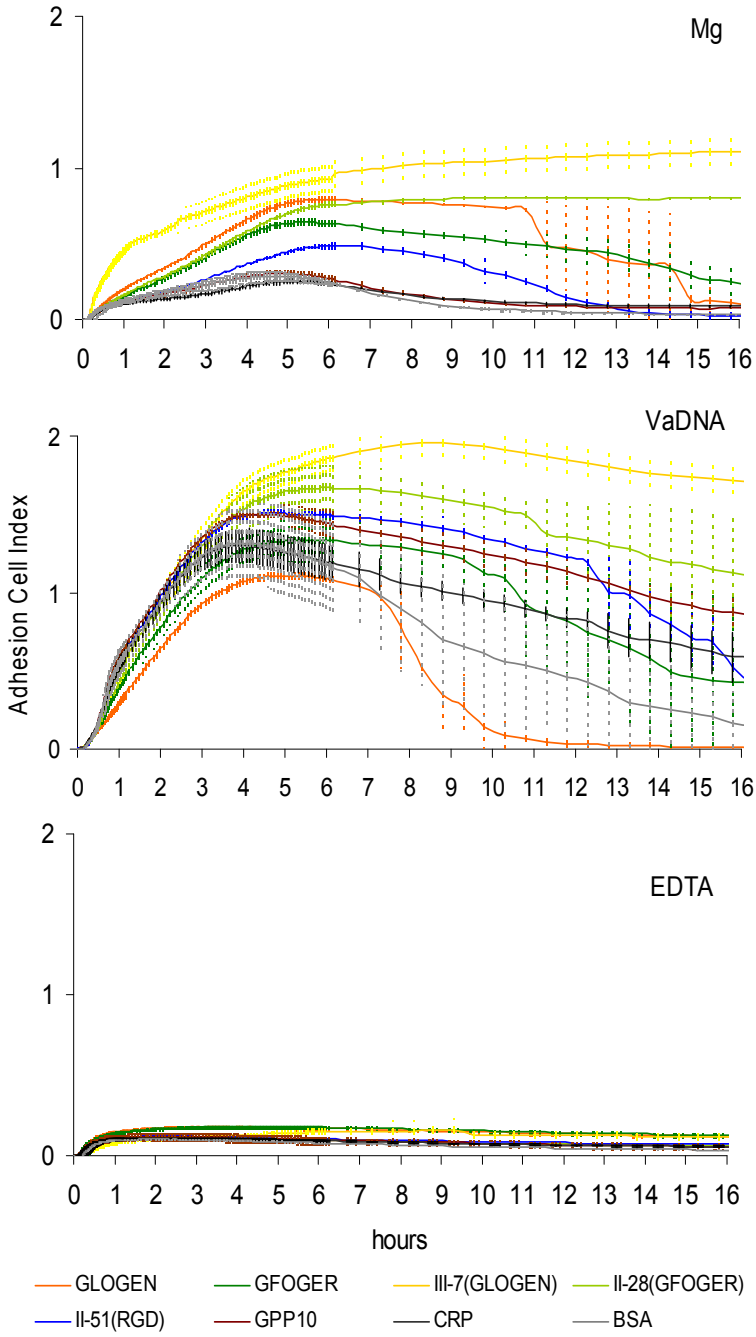
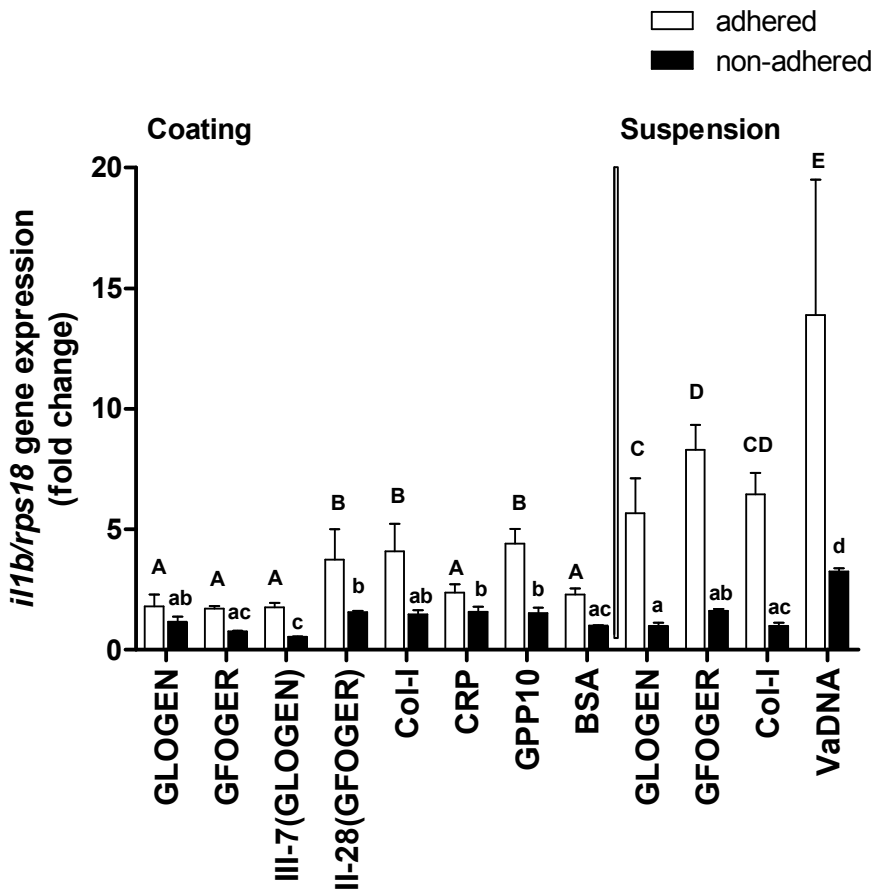

Mg
VaDNA
EDTA
Adhesion Cell Index
hours
GLOGEN
GFOGER
III-7(GLOGEN)
II-28(GFOGER)
II-51(RGD)
GPP10
CRP
BSA

Figure 7


adhered
non-adhered
Coating
Suspension
il1b/rps18 gene expression (fold change)
20
15
10
5
0
A
ab
A
ac
A
c
B
b
B
ab
A
b
B
b
A
ac
C
a
D
ab
CD
ac
E
d
GLOGEN
GFOGER
III-7(GLOGEN)
II-28(GFOGER)
Col-I
CRP
GPP10
BSA
GLOGEN
GFOGER
Col-I
VaDNA

4. IDENTIFICATION OF A GILTHEAD SEABREAM INTEGRIN BETA 1 ISOFORM THAT IS MAINLY EXPRESSED IN TESTIS AND BRAIN, AND IT IS INDUCED DURING SPERMATOGENESIS

Identification of a gilthead seabream integrin beta 1 isoform that is mainly expressed in testis and brain, and it is induced during spermatogenesis

Patricia Castillo-Briceño, Department of Cell Biology and Histology, University of Murcia, Murcia, 30100, Spain.
Sonia Águila-Martínez, Department of Cell Biology and Histology, University of Murcia, Murcia, 30100, Spain.
Marta Arizcun-Arizcun, Oceanographic Centre of Murcia, Spanish Oceanographic Institute (IEO), Puerto de Mazarrón, 30860 Murcia, Spain
José Meseguer, Department of Cell Biology and Histology, University of Murcia, Murcia, 30100, Spain.
Victoriano Mulero, Department of Cell Biology and Histology, University of Murcia, Murcia, 30100, Spain.
Alfonsa García-Ayala, Department of Cell Biology and Histology, University of Murcia, Murcia, 30100, Spain.

Abstract

Extracellular matrix (ECM) is considered to be responsible for the structural properties of tissues and organs. Molecules in the extracellular compartment have a changing composition and organization through development, and interact with diverse cell surface receptors to modulate the transduction of signals for cell growth, differentiation, migration, proliferation, apoptosis, etc. Our previous findings in the teleost fish gilthead seabream (*Sparus aurata* L., Actinopterygii, Teleostei), a marine hermaphrodite fish, have shown gene expression modulation of ECM-related molecules by endocrine and immune stimuli, as well as specific correlations between these molecules during inflammation. In this study, we focused in the analysis by RT-qPCR of the gene expression profile for integrin beta 1 isoform b (ITGB1b) which might play a role in spermatogenesis. In that sense, we analyze its expression profile in the context of the reproductive cycle (RC) and in relation to other ECM-related molecules, including matrix metalloproteases (MMP-2, -9 and -13), tissue inhibitors of MMP (TIMP-2a and -2b), collagen (COL1A1) and ITGB1a. Firstly, we found that ITGB1b was only expressed in testis and brain, and to some extent in endothelial cells, in sharp contrast to ITGB1a, which was ubiquitously expressed. In addition, the expression of ITGB1b in the testis, as well as of other ECM-related molecules, peaked when dramatic tissue remodeling occurs; that is, spermatogenesis and post-spawning stages of both first and second RCs, when specimens are males. Our results suggest a different functionality for the both ITGB1 isoforms detected in gilthead seabream, where ITGB1a would have a constitutive role while ITGB1b seems to have a more specific involvement in reproduction. In that sense this is the first report of an ITGB1 gene whose expression is restricted to endocrine related tissues in vertebrates.

Key words: development, extracellular matrix, integrin beta 1, spermatogenesis, teleost fish, testis, gene expression.

Introduction

After the discovery of integrins and other extracellular matrix (ECM) receptors, the ECM start to be a high interesting field for researchers, not only as an intercellular, structural and tissue scaffold, but as a multifaceted extracellular environment indispensable in the study of diverse cellular processes with an ample functionality [Huxley-Jones et al. 2009, Rozario and DeSimone 2009]. In the extracellular compartment, the molecular composition and organization is actively modulated from fertilization and throughout development [Rozario and DeSimone 2009], affecting the signaling pathways regulatory of cell growth, differentiation, migration, proliferation, polarization and cell death [Hynes 1996, 2009, Tsang et al. 2009].

The interactions between cells and structural ECM components are mediated by a diverse types of receptors like integrins (ITGs), which upon binding may induce the activation of other molecules indispensable for remodeling, inflammation, immune response and wound healing responses, such as: matrikines, matrix metalloproteases (MMPs), tissue inhibitors of MMPs (TIMPs), cytokines and growth factors [Garnotel et al. 2000, Vogel 2001, Tran et al. 2004, Lee et al. 2007, Leitinger and Hohenester 2007, Farndale et al. 2008, Herr and Farndale 2009, Schultz and Wysocki 2009]. In that sense, ablation or misexpression of ECM-cytoplasmic receptors produces several developmental, structural and physiological disease-like phenotypes [Hynes 1996, Lukashev and Werb 1998], although some functions can be supplied by other related genes, as in the case of ITG beta 1 heterodimers (ITGB1s) that show overlapping functions during mice wound healing [Zweers et al. 2007].

ITGs have been described as the main cell surface receptors in eukaryotic organisms [Hynes 2002, Delon and Brown 2007]. This αβ heterodimers family is involved in numerous cellular processes, such as: migration, adhesion, differentiation, proliferation, fibrosis, invasion, leukocytes traffic, haemostasis, tissue repair, remodeling and disease immune response [Sueoka et al. 1997, Datta et al. 2002, Hynes 2002, Larsen et al. 2006, Delon and Brown 2007, Lathia et al. 2009], as well as in specific mechanisms of the reproductive physiology [Bowen and Hunt 2000]. It is also known that modulation of ITG expression patterns is a critical aspect of organogenesis, highly relevant in mechanism like ITG switching in gonadogenesis and adipogenesis [Meighan and Schwarzbauer 2008].

Specifically, COL-ITGs binding plays an important modulator role during embryonic development and organogenesis [Hynes 2002, Johnson et al. 2009], as well as

in wound healing, particularly on MMPs release and cellular adhesion [Pilcher et al. 1999, Grenache et al. 2006, Mócsai et al. 2006, Zhang et al. 2006, Zweers et al. 2007]. ITGs are also able to form complexes with other molecules like the MMPs, allowing them to bind to cell-surface, modulate the intracellular signaling and mediate cell motility by disruption of cell contacts with the ECM [Stefanidakis and Koivunen 2006, Page-McCaw et al. 2007, Butler and Overall 2009]. About ITGB1, it was described to have a relevant role in ectoplasmic specialization in mammals, participating in the adherent and tight junctions between germ and Sertoli cells during spermatogenesis [Siu et al. 2003]. Other ITGs are modulated at transcriptional level by sexual hormones. For example, progesterone increases ITGA4, ITGA5 and ITGB1 subunits expression but does not alter ITGAVB3, which is an ITG heterodimer implicated in implantation and it is inhibited in the presence of estrogens [Bowen and Hunt 2000].

Our previous findings in teleost fish gilthead seabream (*Sparus aurata* L., Actinopterygii, Teleostei) showed that MMP-9, MMP-13, TIMP2a and TIMP2b have a specific expression pattern throughout the first reproductive cycle (RC), and that 17β-estradiol is able to increase the gelatinase activity in testis [Chaves-Pozo et al. 2008a]. In addition, we found a constitutively lower expression of MMP-2, MMP-9, MMP-13, TIMP-2a, TIMP-2b and COL1A1, but higher of ITGB1a in testis compared to other organs in adult fish [Castillo-Briceño et al. 2010]. Recently, it has also been described that ECM-related molecules present a strong correlation between them and can be modulated in response to altered physiological conditions like endometriosis [Salata et al. 2008] and myocardium damage [Ceauşu et al. 2009] in humans, and in response to damage or pathogen associated molecular patterns in fish [Castillo-Briceño et al. 2010].

In the present study we analyze the expression profile of ECM-related molecules during development and adult organs and tissues with immune, endocrine, physiological or structural functions as well as in the testes during the RC stages of gilthead seabream in which the specimens are males, but with special emphasis in the ITGB1 isoforms .

Materials and Methods

Animals

Healthy specimens of gilthead seabream (*Sparus aurata* L., Actinopterygii, Teleostei) were obtained from natural spawning of a captive broodstock in the hatchery facilities of the Spanish Oceanographic Institute (IEO) of Murcia using the “green water”

larval culture technique and following their previously described general protocols for adult fish maintenance (Castillo-Briceño et al. 2009, 2010) and larval growth (Mulero et al. 2007). Animals were fasted for 24 hours before sampling. The experiments performed comply with the Guidelines of the European Union Council (86/609/EU) and the Bioethical Committee of the University of Murcia (Spain) for the use of laboratory animals. Animals were fasted during 24 h before sampling. The experiments performed comply with the Guidelines of the European Union Council (86/609/EU) and the Bioethical Committee of the University of Murcia (Spain) for the use of laboratory animals.

Obtaining of samples from larvae and adult (organs, tissues and cell fractions) specimens

Whole fry of gilthead seabream were sampled at 1-131 days post hatching (dph), while adults of gilthead seabream (1,150 g mean weight) during their testicular involution period were bled and dissected to sample organs and components, according to their relevant immune (gills, thymus, spleen, liver, head kidney, intestine-midgut, peritoneal exudate and blood), endocrine (brain, pituitary, testis and ovary), physiological (kidney and heart-ventricle) or structural (muscle, skin and caudal fin) functions, as previously described [Castillo-Briceño et al. 2010]. In addition, purified fractions of acidophilic granulocytes (AGs) [Sepulcre et al. 2002], macrophages (MCs) [Roca et al. 2006] and endothelial cells (EECs) [Castillo-Briceño et al. 2010] were also analyzed. Testes were sampled in adult animals during the first and second RCs. In all the cases the samples were maintained in TRIzol Reagent (Invitrogen) at -80 ºC following the manufacturer's instructions, until they were processed to RNA extraction.

Analysis of gene expresion

RNA was extracted and then treated with DNase I (1U/µg RNA) amplification grade (Invitrogen) according to the manufacturer's instructions. The RT-qPCR was performed using a SuperScript III ReverseTranscriptase Kit (Invitrogen) to synthesize first strand cDNA from 1 µg of total RNA, and SYBR Green PCR Core Reagents (Applied Biosystems) with an ABI PRISM 7500 instrument (Applied Biosystems) according to the respective manuals. For each mRNA, gene expression was normalized to the ribosomal protein S18 content in each sample using the comparative Cq method (ΔΔCq). The primers used are shown in Table 1. In all cases, each PCR was performed by triplicates and

repeated with at least two independent samples, as previously described [Castillo-Briceño et al. 2009, 2010].

Statistical analysis

Data of whole organs, tissues and cells sampled were analyzed by a one-way analysis of variance (ANOVA) and a Bonferroni's post-test for grouped data sets. Larvae data correlation between the ECM-related molecules and developmental progress (dph) was assessed by Pearson's test. EMC related molecule correlations among them in larvae and in testis during RCs were analyzed by a Pearson's matrix correlation test, which calculates r for each pair of data sets. All the analyses according to the recommendations of the statistical software used (Prism 5 for Windows version 5.00) in accordance to the number of conditions, factors, replicates, repeated measures, distribution, etc. In all the cases significance was considered for $p < 0.05$.

Results

The expression of ECM-related molecules correlates with developmental stages

The gene expression profiles of the ECM-related molecules studied showed that the expression levels of MMP-2, -9, TIMP-2a, -2b and COL1A1 were strongly correlated (Pearson $r > 0.70$, $p < 0.05$) during development in both larval (1-90 dph) and juvenile (90-140 dph) stages (Figure 1, Table 2). Furthermore, it was found different correlation patterns among these genes where TIMP-2b correlates with all the others (Pearson $r > 0.65$, $p < 0.05$). There were also an initial high expression, and a strong correlation, between MMP-2, TIMP-2a and COL1A1 (Pearson $r > 0.92$, $p < 0.001$), but not with MMP-9 (Table 2), likely because of only MMP-2 and TIMP-2a showed a peak at 47 dph. Notably, MMP-13 and ITGB1a were non-significantly correlated to any analyzed gene or to the developmental stage (Figure 1, Table 2) and they showed isolated peaks at 12 and 1-4 dph, respectively.

ITGB1b gene expression is restricted to brain, testis and endothelial cells

ITGB1b mRNA was non-detected in whole fry during development. Further, analysis in adult specimens for different organs and tissues showed that the ITGB1b expression was mainly restricted to the testis and the brain, although at lower levels in the later, and in minimal levels in spleen, heart-ventricle, skin, muscle, ovary and peritoneal

exudate. It was also found that ITGB1b expression in EECs was higher than the minimal levels of the last mentioned organs (Figure 2). In the other analyzed organs and tissues (gills, thymus, liver, head kidney, intestine-midgut, pituitary, kidney, caudal fin and blood), and cell fractions (AGs and MCs) ITGB1b was not detected.

The expression pattern of ITGb1b throughout the RC is different to the other ECM-related molecules

In testis, during the second RC, the gene expression pattern of MMP-2, -9 and -13 were closely correlated among them (Pearson $r > 0.87$, $p \leq 0.001$) but also to TIMP-2a (Pearson $r > 0.76$, $p \leq 0.01$) and COL1A1 (Pearson $r > 0.71$, $p < 0.05$), with a sharp increase in post-spawning (Figure 3, Table 3). MMP-13 also correlated (Pearson $r > 0.67$, $p < 0.05$) with TIMP2b, COL1A1 and ITGB1a, showing weaker peaks during spermatogenesis (Figure 3, Table 3). While ITGB1b showed a specific peak in spermatogenesis and was correlated only with ITGB1a (Pearson $r > 0.75$, $p < 0.05$) (Figure 3, Table 3). In the first RC, ITGB1b also showed a single sharp peak in the spermatogenesis stage but did not correlate to ITGB1a, which was correlated with COL1A1 (Pearson $r = 0.76$, $p < 0.05$) and reached their maximal levels at the beginning of the spawning stage (data not shown).

Discussion

The correlation between MMP-2, -9, TIMP-2a, -2b and COL1A1 with the developmental stages and their high initial levels after hatching coincide with the reported tendency for growth related molecules in gilthead seabream, such as insulin-like growth factor 1 transcripts (IGF-1a, b, c) [Tiago et al. 2008], osteonectin (OSN) [Estêvão et al. 2005], cortisol and thyroid hormones (T3 and T4) [Szisch et al. 2005] as well with the robust expression of innate immune genes [Mulero et al. 2008]. It suggests well coordinated interaction and regulation of the ECM-related molecules during seabream growth and organogenesis, which agrees with the general requirement of a fine-tune balance of MMPs/TIMPs for a normal development in vertebrates [Rolland et al. 1998, Zhang et al. 2003, Crawford and Pilgrim 2005, Smith et al. 2006, Bednarek et al. 2009, Wyatt et al. 2009] and activation of immune pathways [Werb 1997, Mott and Werb 2004, Stevens et al. 2009].

The expression increment at 47 dph for the most of the ECM-related molecules observed concurs with the rising levels of IGF-1a and IGF-1c [Tiago et al. 2008], cortisol [Szisch et al. 2005] and adaptive immunity genes [Mulero et al. 2008] described for gilthead seabream, and correspond with its ossification of the skeletal structures processes [Estêvão et al. 2005] and the accumulation of erythrocytes in the spleen and infiltration of AGs in the intestine [Mulero et al. 2007], an scenario that implies strong remodeling and morphogenesis conditions probably related to the transition from larvae to juvenile stages. MMP-13 expression peaks at 12 dph, coinciding with the ending phase of the cartilage development and the increase of OSN that occurs previously to the ossification of cephalic structures and pectoral fins [Estêvão et al. 2005], pointing to a particular involvement in the ossification process, as widely described for mammals [Inada et al. 2004, Stickens et al. 2004, Krane and Inada 2008, Takaishi et al. 2008]. In the same way, ITGB1a higher levels at 1 and 4 dph coincides with chondrogenesis starting in the same structures [Estêvão et al. 2005], in agreement to mammalian ITGB1 heterodimers signaling during cartilage development and MMP-13 expression induced by COL [Ivkovic et al. 2003, Ronzière et al. 2005, Djouad et al. 2007, Krane and Inada 2008]. In that way, MMP-13 and ITGB1a in the gilthead seabream appear to have a relevant role during larval skeletal development, which is not related to general coordination between the other ECM-related molecules.

The absence of ITGB1b gene expression during non-adult development stages and its particular profile in organs of adults outlines a different role to the described for the other ECM-related molecules in tissue remodeling and regeneration in gilthead seabream [Castillo-Briceño et al. 2010]. ITGB1a appears to have a more constitutive role in gilthead seabream, being expressed since the first dph and in all the organs of adult gilthead seabream [Castillo-Briceño et al. 2010], converging with the expression profile of zebrafish (*Danio rerio*) ITGB1 paralogs which are all expressed after 24 h post-fertilization and in all the adult organs [Mould et al. 2006]. In that sense, it is interesting that gilthead seabream ITGB1b has a restricted expression profile that not correspond to any of the zebrafish ITGB1 paralogs described [Mould et al. 2006]. Therefore, ITGB1a would be sharing the functions of the single ITGB1 subunit found in higher vertebrates, as suggested for zebrafish ITGB1a and ITGB1b [Mould et al. 2006], while ITGB1b may have more specialized or novel roles.

ITGB1b expression in EECs could be part of a refinement and/or complement of ITGB1a signaling, to fulfill the multifaceted roles of the single ITGB1 in mammalian endothelium [Sueoka et al. 1997, Bowen and Hunt 2000, Al-Jamal and Harrison 2008,

Lathia et al. 2009], where is involved in processes like adhesion of leukocytes to endothelial cells [Kuijpers et al. 1990] and has an important pleiotropic functionality during vasculature development key steps, such as endothelial cell maturation, migration and elongation [Malan et al. 2010]. EECs might be responsible for the very low expression of ITGB1b in spleen, heart, ovary, muscle, skin and peritoneal exudate. In mice brain, it has been described that ITGB1 is only expressed in blood vessels, where it plays a pivotal role in neurovascular remodeling [Lathia et al. 2009], while in rats was also detected in neurons and glia [Pinkstaff et al. 1998, Gall et al. 2003]. In fish, it has not been described so far the localization of ITGB1 isoforms in brain. However, its higher expression levels in brain compared to isolated EECs suggests, as in testes, that EECs would not be the only source of ITGB1b in this organ.

The expression dynamics and specific correlations between the ECM-related molecules in testis appear to be part of the dramatic changes occurring during the RC and are in agreement with the suggested fine tuning of this molecules in tissue remodeling and other physiological processes in vertebrates [Stevens et al., 2009, Castillo-Briceño et al. 2010]. The MMPs, TIMPs and COL1A1 increase in post-spawning matches with the expression profile of these TIMPs during the first RC in gilthead seabream, but not with the higher expression levels of MMP-9 and -13 before spawning in this RC [Chaves-Pozo et al. 2008a]. This difference could be explained by complex tissue remodeling after the second RC that leads to testicular involution, which includes (i) diminished gonadosomatic index [Liarte et al. 2007, Chaves-Pozo et al. 2008c], (ii) elevated expression levels of several inflammatory related molecules, such as interleukin-8, tumor necrosis factor α and transforming growth factor β1 [Chaves-Pozo et al. 2008b], and (iii) the infiltration of AGs probably favored by an overexpression of MMPs [Chaves-Pozo et al. 2005, Liarte et al. 2007]. In addition, MMP-13 and TIMP-2b elevated expression in spermatogenesis concurs with the described for these molecules in the first RC [Chaves-Pozo et al. 2008a] and coincides with germ and Sertoli cell proliferation [Chaves-Pozo et al. 2005].

Regulation of ITG gene expression affects their repertoire in the cell surface and alters their proportions, acting as an ITG switching to provide an exquisite control over cell rearrangements during tissue morphogenesis and remodeling, also in relation to other ECM related molecules [Meighan and Schwarzbauer 2008]. In that sense, our data for ITGB1a and its strong correlations with the ECM related molecules, especially with COL1A1 in both RCs suggest a role for this isoform more related to the general tissue remodeling in testes, while ITGB1b would be more involved in specific processes related to the

spermatogenesis, like the described implication of mammalian ITGB1 in adherent and tight junction formation of Sertoli and developing germ cells required for ectoplasmic specialization during spermatogenesis [Siu et al. 2003] and its generalized relevant role in mammalian reproduction [Sueoka et al. 1997, Bowen and Hunt 2000, Siu et al. 2003, Johnson et al. 2009]. Moreover, since the increased expression of ITGB1b coincides with the highest concentrations of testosterone and 11-ketotestosterone in plasma [Chaves-Pozo et al. 2008c], it is tempting to speculate that this isoform could have a more specific contribution to endocrine and reproductive pathways in a refinement of the ITGB1a functions, supported by ITGB1b minimal or null expression in the other organs, and its lack of correlation to the other ECM-related molecules.

Summarizing, we propose that the ITGB1 isoforms detected in gilthead seabream would have different activities: ITGB1a would perform mainly as a constitutive receptor in the context of the ECM environment and structural dynamics, while ITGB1b would entail a specialized role that could be modulated by steroid hormones during the RCs. Whatever the outcome, this is the first report of an ITGB1 gene whose expression is restricted to endocrine related tissues in vertebrates. Further, we are performing additional experiments *in vivo* to analyze the possible effect of sexual hormones on the expression of ITGB1b in testes of adult fish, thus we used testosterone as an androgenic stimuli and 17α-ethynylestradiol as an androgen disruptor, sampling at different periods of exposure for its posterior processing.

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Competing Interests

The authors have declared that no competing interests exist.

Abbreviations

AG, acidophilic granulocytes; Cq, quantification cycle; dph, days post hatching; EEC, endothelial cells; MC, macrophages; RC, reproductive cycle.

Figure Legends

Figure 1

Gene expression pattern of ECM-related molecules in gilthead seabream correlates with larvae and juveniles developmental stages. The mRNA levels of the indicated ECM related genes were determined by RT-qPCR in amplification products obtained from pools of whole fish of at least six fish at the indicated ages. Gene expression is normalized against rps18. Each bar represents the mean ±S.E. of triplicate analysis trough development in days post hatching (dph). All genes excepting MMP-13 and ITGB1a were significantly correlated to dph increase according to Pearson's test ($p < 0.05$). Different letters denote statistically significant differences between the groups according to Tukey's test from a one-way ANOVA ($p < 0.05$).

Figure 2

ITGB1b is mainly restricted to testis, brain and endothelial cells (EECs) in adult specimens of gilthead seabream. The mRNA levels of ITGB1b were determined by RT-qPCR in amplification products obtained from two pools of at least eight fish each one, sampled during the testicular involution stage of the reproductive cycle. Gene expression is normalized against rps18 and relative to testis. Each bar represents the mean ±S.E. of triplicate analysis. Different letters denote statistically significant differences between the groups according to Tukey's test from a one-way ANOVA ($p < 0.05$).

Figure 3

ECM-related molecules gene expression pattern have specific correlations through the second reproductive cycle in testis of adult gilthead seabream. The mRNA levels were determined by RT-qPCR in amplification products obtained from pools of whole testes of at least eight healthy fish at the indicated months and reproductive stages. Gene expression is normalized against rps18. Each curve represents the mean ±S.E. of triplicate analysis. Different letters denote statistically significant differences between the groups according to Bonferroni's post-test for a two-way ANOVA ($p<0.05$). SG = spermatogenesis, S = spawning, PS = post-spawning, TI = testicular involution.

Tables

Table 1

Primer sequences used for gene expression analysis. The gene symbols followed the Zebrafish Nomenclature Guidelines (http://zfin.org/zf info/nomen.html).

Gene	**Accession number**	**Primer name**	**Sequence (5'→3')**
rps18	AM490061	F	AGGGTGTTGGCAGACGTTAC
		R	CTTCTGCCTGTTGAGGAACC
mmp2	FN649419	F1	ACTATGACCGCGACAAGTCC
		R1	GTAACCTTGGTCGGGACAGA
mmp9	AM905938	F1	GGGGTACCCTCTGTCGATTT
		R1	CCTCCCCAGCAATATTCAGA
mmp13	AM905935	F	CGGTGATTCCTACCCATTTG
		R	TGAGCGGAAAGTGAAGGTCT
timp2a	AM905937	F	CAAAGGTGGTGGGAGAGAAA
		R	TTGACGTCCAGGGTAACTCC
timp2b	AM905936	F	ATGTCGTTATCAGGGCGAAG
		R	AGAAGTGGGAGCGGTGTAGA
col1a1	DQ324363	F2	GCTCTCAGCCAGAGGATGTC
		R2	TGTAGGCGATGCTGTTCTTG
itgb1a	FN649420	F2	AAGGGAGACGAGTTCAATCGGT
		R1	CACCAGCAGACGAGTCACAT
itgb1b	FN649421	F1	AACGGAAGCCTCTTCACAGA
		R1	CACCAGCAGACGAGTCACAT

Table 2

ECM-related molecules gene expression correlations in larvae and juveniles of gilthead seabream (*Sparus aurata*).

	mmp2	***mmp9***	***mmp13***	***timp2a***	***timp2b***	***col1a1***	***itgb1a***
mmp2		**0.41**	**0.08**	**0.97***	**0.75***	**0.93***	**0.45**
mmp9	0.186		**0.22**	**0.48**	**0.65***	**0.43**	**-0.29**
mmp13	0.802	0.493		**0.22**	**0.05**	**0.06**	**-0.13**
timp2a	<0.001	0.116	0.489		**0.78***	**0.92***	**0.43**
timp2b	0.005	0.023	0.885	0.003		**0.81***	**0.12**
col1a1	<0.001	0.164	0.863	<0.001	0.001		**0.43**
itgb1a	0.141	0.354	0.685	0.164	0.712	0.167	
Total correlated genes	3	1	0	3	4	3	0

r = correlation coefficient (bold style numbers)
p = p value (two-tailed)
* Significant **r** ($p > 0.05$)

Table 3

ECM-related molecules gene expression correlations in testis of adult gilthead seabream (*Sparus aurata*) trough the second reproductive cycle.

	mmp2	***mmp9***	***mmp13***	***timp2a***	***timp2b***	***col1a1***	***itgb1a***	***ltgb1b***
mmp2		**0.98***	**0.90***	**0.88***	**0.55**	**0.71***	**0.40**	**0.01**
mmp9	<0.001		**0.87***	**0.83***	**0.54**	**0.72***	**0.36**	**-0.06**
mmp13	<0.001	0.001		**0.76***	**0.81***	**0.74***	**0.67***	**0.34**
timp2a	0.001	0.003	0.010		**0.42**	**0.44**	**0.36**	**-0.09**
timp2b	0.100	0.104	0.004	0.228		**0.57**	**0.63***	**0.47**
col1a1	0.021	0.018	0.014	0.202	0.086		**0.52**	**0.47**
itgb1a	0.255	0.305	0.034	0.302	0.049	0.122		**0.75***
itgb1b	0.968	0.860	0.333	0.815	0.174	0.166	0.012	
Total correlated genes	4	4	6	3	2	3	3	1

r = correlation coefficient (bold style numbers)
p = p value (two-tailed)
* Significant **r** ($p > 0.05$)

Figure 1

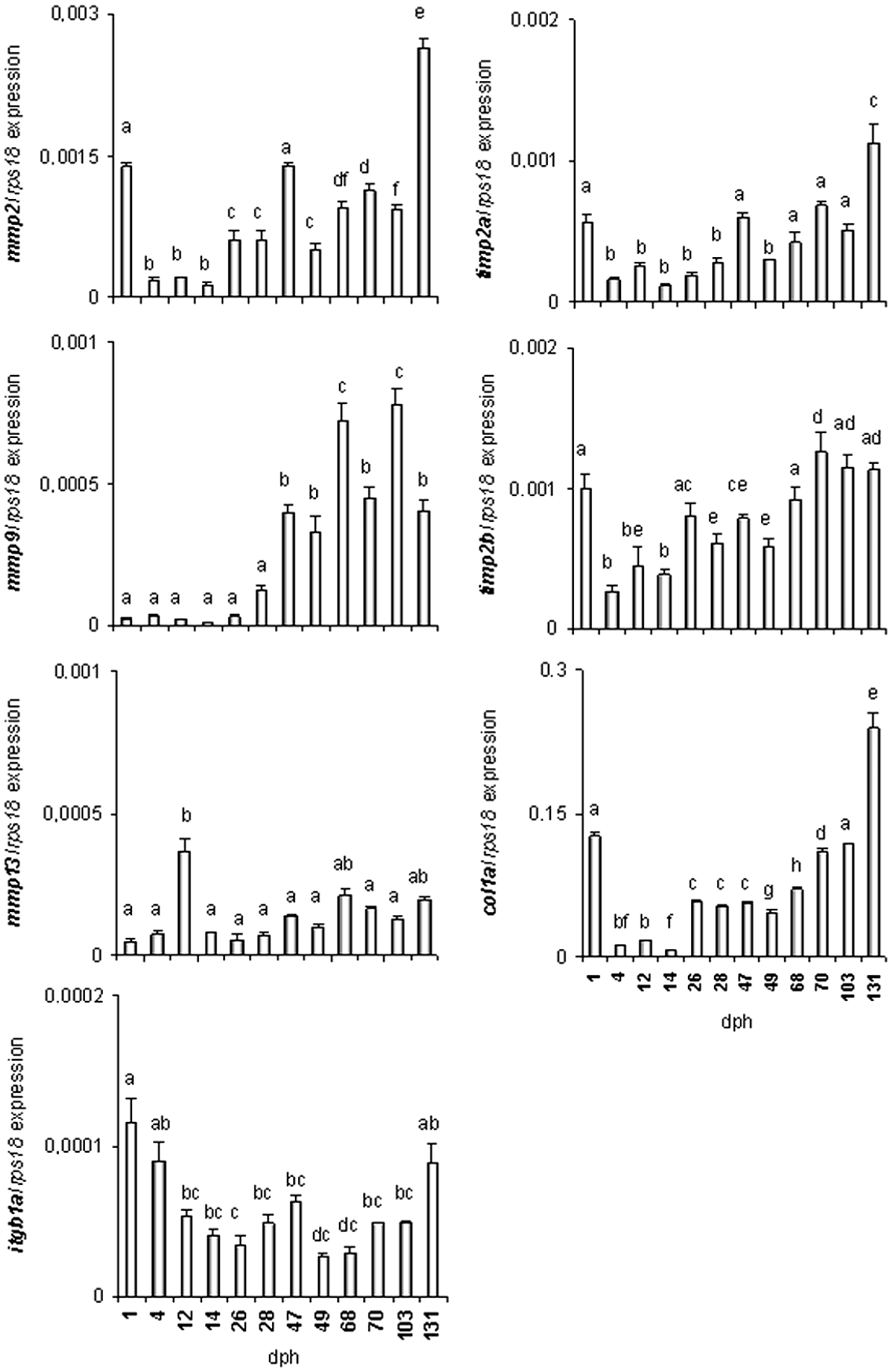

mmp2/rps18 expression
0,003
0,0015
0
a b b b c c a c df d f e
timp2a/rps18 expression
0.002
0.001
0
a b b b b b a b a a a c
mmp9/rps18 expression
0,001
0,0005
0
a a a a a a b b c b c b
timp2b/rps18 expression
0.002
0.001
0
a b be b ac e ce e a d ad ad
mmp13/rps18 expression
0.001
0,0005
0
a a b a a a a a ab a a ab
col1a/rps18 expression
0.3
0.15
0
a bf b f c c c g h d a e
itgb1a/rps18 expression
0.0002
0,0001
0
a ab bc bc c bc bc dc dc bc bc ab
1 4 12 14 26 28 47 49 68 70 103 131
dph

Figure 2

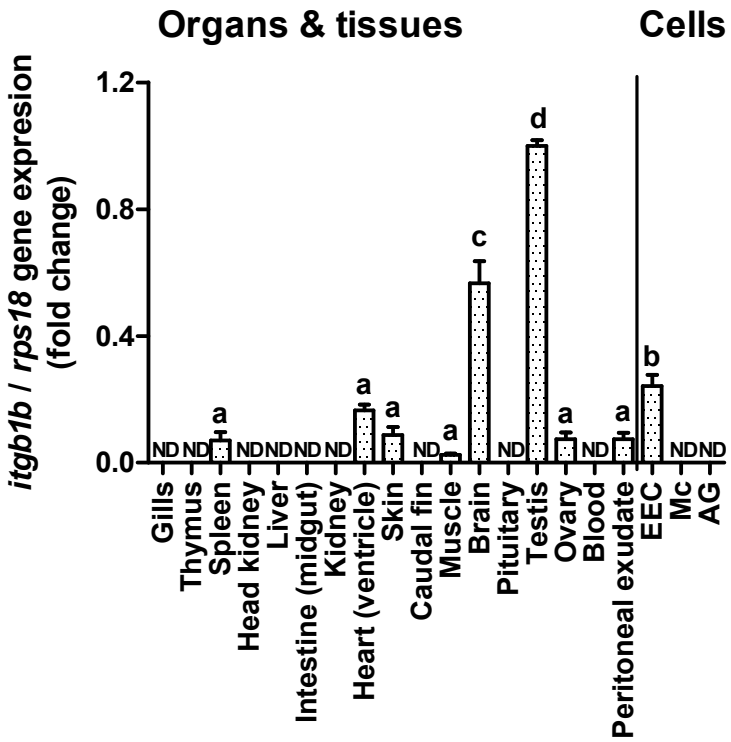
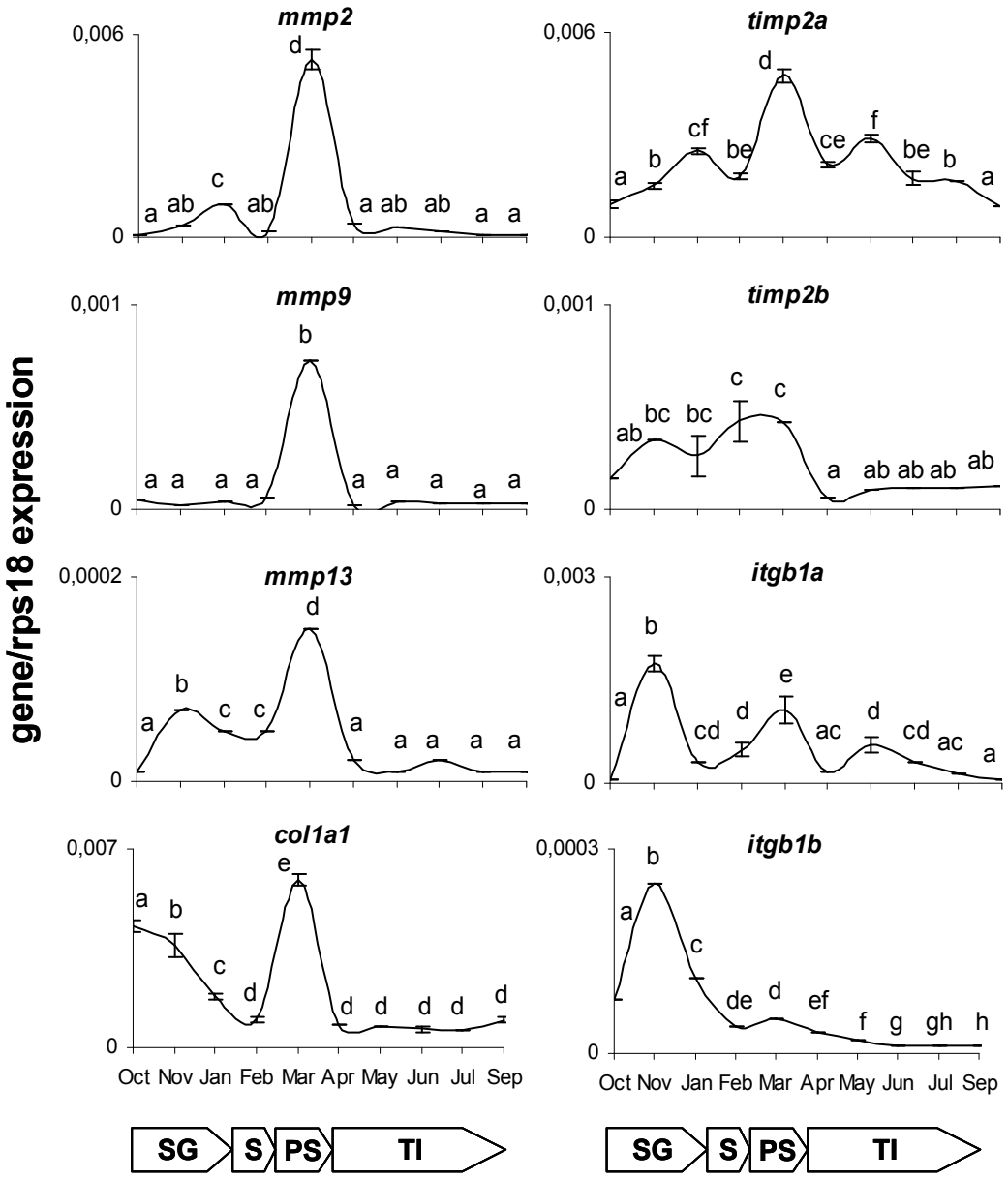

Organs & tissues
Cells
itgb1b / rps18 gene expresion (fold change)
1.2
0.8
0.4
0.0
ND ND a ND ND ND ND a a ND a c ND d a ND a b ND ND
Gills
Thymus
Spleen
Head kidney
Liver
Intestine (midgut)
Kidney
Heart (ventricle)
Skin
Caudal fin
Muscle
Brain
Pituitary
Testis
Ovary
Blood
Peritoneal exudate
EEC
Mc
AG

Figure 3


gene/rps18 expression
mmp2
0,006
0
a ab c ab d a ab ab a a
timp2a
0,006
0
a b cf be d ce f be b a
mmp9
0,001
0
a a a a b a a a a a
timp2b
0,001
0
ab bc bc c c a ab ab ab ab
mmp13
0,0002
0
a b c c d a a a a a
itgb1a
0,003
0
a b cd d e ac d cd ac a
col1a1
0,007
0
a b c d e d d d d d
itgb1b
0,0003
0
a b c de d ef f g gh h
Oct Nov Jan Feb Mar Apr May Jun Jul Sep
SG S PS TI
SG S PS TI

ANEXOS

1 Participación en congresos y comunicaciones correspondientes al trabajo de tesis

9th International Congress on the Biology of Fish. Barcelona, España. Jul, 2010

Specific collagen motifs promote adhesion and induce a pro-inflammatory response in fibroblasts of the teleost fish gilthead seabream. **P. Castillo-Briceño,** R.W. Farndale, A. García-Ayala, V. Mulero. Póster.

Evo-Devo Meets Marine Ecology: New Frontiers in Ocean Science through Integrative Biology. EMBO Workshop. Sant'Angelo d'Ischia, Italia. Oct, 2009

A possible role of extracellular matrix molecules in the immune response of gilthead seabream *Sparus aurata* L**. P. Castillo-Briceño**, I. Cabas, S. Liarte, J. Meseguer, A. García-Ayala, V. Mulero. Póster.

11th Congress of the International Society of Developmental and Comparative Immunology - ISDCI 2007. Praga, República Checa. Jul, 2009

Identification of the collagen motifs recognized by fibroblasts and immune cells from the bony fish gilthead seabream. **P. Castillo-Briceño**, S. W. Hamaia, V. Mulero, R. W. Farndale and A. García-Ayala. Comunicación oral.

Expression of extracellular matrix molecules in immune cells and tissues of the gilthead seabream. **P. Castillo-Briceño**, S. Liarte, I. Cabas, J. Meseguer, A. García-Ayala, V. Mulero. Comunicación oral.

Encuentro Científico Internacional - ECI 2007 invierno. Lima, Perú. Ago, 2007

La biología molecular en la vida marina. **P. Castillo-Briceño.** Comunicación oral - Sesión Plenaria.

El colágeno es un potente activador de los fagocitos de peces. **P. Castillo-Briceño**, Mª Pilar Sepulcre, Elena Chaves Pozo, José Meseguer, Alfonsa García Ayala y Victoriano Mulero. Comunicación oral.

XII Congreso de la Sociedad Española de Biología Celular. Pamplona, España. Jul, 2007

El colágeno es un potente activador de los fagocitos de peces. **P. Castillo-Briceño**, Mª Pilar Sepulcre, Elena Chaves Pozo, José Meseguer, Alfonsa García Ayala y Victoriano Mulero. Comunicación oral.

2 Participación en congresos y comunicaciones correspondientes a colaboraciones durante el trabajo de tesis

9th International Congress on the Biology of Fish. Barcelona, España. Jul, 2010

Seasonal changes in the expression of immune related molecules in the teleost fish gilthead seabream. M. Sánchez-Hernández, **P. Castillo-Briceño**, E. Abellán, A. García-Alcázar, A. García-Ayala, V. Mulero. Póster.

First Symposium of the European Organisation of Fish Immunology (EOFFI). Viterbo, Italia. May, 2010

Testosterone modulates the immune response in vivo of the teleost fish gilthead seabream. S. Águila-Martínez, **P. Castillo-Briceño**, J. Meseguer, V. Mulero, A. García-Ayala. Póster.

13th International Congress of Immunology - Immuno Rio 2007. Río de Janeiro, Brasil. Ago, 2007

Leukocytes and cytokines in the gilthead seabream (*Sparus aurata*) testis. J. Meseguer, E. Chávez Pozo, S. Liarte, A. García Alcázar, **P. Castillo-Briceño**, L. Fernández, V. Mulero y A. García Ayala. Póster.

Encuentro Científico Internacional - ECI 2007 invierno. Lima, Perú. Ago, 2007

Sustitución del cuidado parental en el cultivo del combatiente de SIAM (Betta splendens, Reagan 1910). K.F. Navarrete-Mier y **P. Castillo-Briceño**. Comunicación oral.