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Survivin and Cyclooxygenase-2 are co-expressed in human and mouse colon carcinoma and in terminally differentiated colonocytes

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Summary. In the evolution of colon rectal cancer (CRC) the imbalance between cell proliferation and apoptosis is considered one of the prominent causes of tumor induction and/or progression. In order to establish the role of anti apoptotic proteins in colon cancer development, we studied with immunohistochemical techniques the expression of Survivin in a mouse model of colon carcinogenesis induced by 1,2-dimethyl-hydrazine treatment.

In this mouse model Survivin was over-expressed during tumor development, showing a distribution mimicking that described in the correspondent human malignancies. We also correlated Survivin distribution with COX-2 and ß-Catenin expression patterns.

The co-localization of COX-2/ß-Catenin/Survivin in the same epithelial cells in tumor samples lends credence to possible *in vivo* regulatory effects of COX-2 and ß-Catenin on the intracellular Survivin levels in mouse and human colon cancer.

Key words: Colon cancer, Mouse, 1,2-dimethyl-hydrazine

Introduction

During the last decade a highly conserved family of apoptosis regulatory molecules named IAPs (inhibitors of apoptosis), which are distinct from the Bcl-2 family, have been described first in *Baculovirus* (Crook et al., 1993) and shortly after in a variety of organisms and also in mammals (O'Driscoll et al., 2003).

Numerous studies on apoptosis have shown that this mechanism plays a central role not only in the correct tissue homeostasis and developmentally regulated morphogenesis (Vaux et al., 1994), but also in the continuous elimination of mutated or virally transformed cells. Several studies have proved that disturbance of apoptosis can potentially underlie tumor promotion and/or progression (Gianani et al., 2001; Yu et al., 2002). Survivin is a new member of this IAP family (Ambrosini et al., 1997; Adida et al., 1998; Tamm et al., 1998) that seems to be highly expressed in several human tumors, e.g. gastric, colon and endometrial cancer (Gianani et al., 2001; Yu et al., 2002; Kim et al., 2003). Survivin is a peculiar landmark of neoplastic transformation apparently not linked to the histopathological connotations or grading of tumor formations. During colon cancer progression, Survivin apparently has the dual function of suppressing tumor cell apoptosis and regulating the cell-cycle. This peculiarity concurs to both accelerate proliferative activity and inhibit programmed cell death (Kawasaki et al., 1998; Gianani et al., 2001; O'Driscoll et al., 2003).

While in embryonic tissues high levels of Survivin expression are commonly reported, terminally differentiated tissues are often negative or show low expression, restricted to their proliferative compartment (stem cells) (O'Driscoll et al., 2003), whereas high levels of Survivin expression are often observed during cancer

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Abbreviations: AOM, azoxymethane; CRC, colon rectal cancer; DMH, 1,2-dimethylhydrazine; IAP, inhibitors of apoptosis; PGE2, Prostaglandin E2; ACF, aberrant crypt foci; hTDM, human mucosa distant from the tumor; hTPM, human tumor proximal mucosa.

formation. As a consequence of these observations, Survivin is presently considered a potential target for human cancer therapy (Altieri, 2003).

Mechanisms proposed for Survivin transcriptional regulation (Li and Altieri, 1999) suggest that its involvement in colorectal cancer cell proliferation and resistance to apoptosis are linked to TCF/ß-Catenin signaling (Kim et al., 2003). Another interesting mechanism for Survivin intracellular stabilization was recently described as a COX-2 dependent PGE2 (Prostaglandin E2) mediated by lowering Survivin ubiquitination levels in non-small cell lung cancer (Krysan et al., 2004).

Considering that COX-2 can also be up-regulated in human colorectal adenomas, adenocarcinomas (Eberhart et al., 1994; Elder et al., 2002) and in 1,2-dimethylhydrazine-induced colon carcinogenesis in rodents (Shao et al., 1999; Dong et al., 2003) we decided to investigate Survivin translational control and/or protein stabilization mechanisms *in vivo* during colon tumor progression in humans and in mice. Survivin, COX-2 and ß-Catenin expression patterns were studied on serial sections from human colon cancer samples at different stages and from similar mice colon tumor samples induced by repetitive treatment of a susceptible mice strain (A/J) with the alkylating carcinogens 1,2-dimethylhydrazine (Nambiar et al., 2003). This mouse model reproduces the typical morphological alterations of the human sporadic form of colon cancer and a number of its genetic abnormalities (Corpet and Pierre, 2003).

In this paper we describe for the first time Survivin up-regulation in 1,2-dimethylhydrazine induced colon tumorigenesis in mice and we show that Survivin and COX-2 are co-localized in mice and in human colon adenocarcinoma samples. We have also compared the pattern of these genes in the human and mouse ACF (aberrant crypt foci) and non-ACF colon mucosa, focusing on the similarities and/or differences between these two systems.

Materials and methods

Chemicals

Animals were treated with 1,2-dimethylhydrazine (DMH), purchased from SIGMA (CAT. D16,180-2, Missouri, USA) for the induction of the colon cancer.

For the immunohistochemistry the following rabbit polyclonal antibodies were used: anti-COX2 (No 160126 CAYMAN chemical, Ann Arbor, MI), used at 1:300 dilution; anti-ß-Catenin (RB-090-P0, NEOMARKERS, Fremont, USA), 10mg/ml; anti-Survivin (cat. #500-201 Novus Biologicals, Littleton, CO, USA), 1:250. The specificity of this latter antibody has been shown in several papers (Fortugno et al., 2002; Yu et al., 2002) both for Western blot and immunohistochemical applications.

The anti rabbit antibody used for immunohistochemistry was Anti-Rabbit BA-1000 (VECTOR, Burlingame USA); that used for Western Blot was Anti-Rabbit A6154 (SIGMA, Missouri USA). As amplification system the anti-Rabbit ABC complex (VECTASTAIN ABC Kit, PK-6100) was used and for the staining of the sections the diaminobenzidine staining kit (Peroxidase substrate Kit, SK-4100), both purchased from VECTOR (Burlingame, USA).

Animal treatment

Six weeks old A/J mice were purchased from the Charles-River (Milano, Italy), housed 5 animals/cage in a temperature controlled light cycled room (12h light/dark) and fed with PURINA Laboratory chow 5001 *ad libitum*. Animals were injected *in intraperitoneum* (IP) once a week for 6 weeks with 20 mg DMH. The carcinogen was dissolved in Tris-HCl and buffered with 1N NaOH at pH6.5.

Animals were euthanized 5, 9, 15, 26 and 32 weeks after the end of the treatment.

All animal procedures were conducted in conformity with national and international laws and policies for animal care and use.

Human samples

Tissue Micro Array containing human colon cancer samples at different stages of differentiation (Grading G1, G2, G3) came from Tristar Technology Group LLC (Bethesda, MD, USA) and nineteen human colon tumors embedded in paraffin (Grading G1, G2, G3) and the matching normal mucosa of a sub set of them were obtained from the Department of Human Pathology of the Università degli Studi di Roma "La Sapienza" and from The Department of Anatomic Pathology, Ospedale S.Giovanni-Addolorata, Rome, Italy (Table 1). The samples were all anonymous and standard ethical protocols were adopted in order to protect the privacy of the patients.

Tissue Micro Arrays sampling a variety of normal mouse tissues were obtained from Chemicon (TMA2901, Temecula, USA) (Table 2).

Immunohistochemical staining for Survivin

Immunohistochemistry was performed essentially as described in Lazzaro et al. (1991) briefly, tissues were fixed in 10% buffered formalin and embedded in paraffin. 5 μm microtome sections were cleared in xylol and re-hydrated, the unmasking procedure was carried out by immerging the samples in DAKO antigen retrieve solution 10X (Cod. No. S1699, DAKO Cytomation, Carpinteria, USA) at 99°C for 40 minutes; after rinsing in PBS, the sections were covered with blocking solution, 15 μl goat serum in 1 ml PBS triton 0.1% (PBS TRN), for 20 minutes at room temperature. Without

additionally rinse they were covered with the appropriate antibody and incubated O/N at 4°C.

Then sections were rinsed in PBS TRN and PBS and incubated with anti Rabbit Antibody at the dilution of 1:200 for 30 minutes at room temperature.

Three washing steps followed with respectively: PBS TRN, PBS H_2O_2 3% (3 minutes) and PBS. For signal amplification analysis, sections were incubated with Anti Rabbit ABC Complex for 30 minutes at room temperature, then rinsed in PBS and stained with the diaminobenzidine staining kit for about 1 minute. Sections were dehydrated and mounted with Entellan (OB 043984 Merck KGaA, Germany).

Western blot

Colon tissues were homogenized in 500 μl RIPA Buffer, containing 1% Nonidet P-40 (Fluka, Neu-Ulm, Germany), 0.5% sodium deoxicholate, 0.1% SDS, COMPLETE protease Inhibitor Cocktail Tablets (cat#1697498 ROCHE, Mannheim, Germany) in 1xPBS.

Protein concentrations were measured by BCA Kit (Prod.#23225 BCA Protein Essay Kit, PIERCE Chemical Co., Rockford, IL USA) Twenty micrograms of protein were loaded per lane, separated by 12% SDS-polyacrylamide gel (NuPAGE Novex 12% Bis-Tris Gel NP0341BOX, INVITROGEN, Carlsbad, CA 92008 USA) and transferred onto equilibrated Cellulosenitrate membrane (Protran BA 85 Cellulosenitrate 401189, SCHLEICHER&SCHUELL, Dassel, Germany) by electroblotting. Membrane was blocked by 5% non-fat dry milk, 1% BSA and then incubated with an antibody against Survivin (1μg/ml) at 4°C O/N. After secondary antibody incubation (1:3000, 1h) Survivin was detected by enhanced chemiluminescence (ECL Western Blotting Detection Reagent RPN2106, AMERSHAM, Buckinghamshire, England).

Results

Survivin induction after 1,2-dimethylhydrazine treatment

Mouse samples were collected at different time points from the start of induction with DMH: 5, 9, 15, 26, and 32 weeks. We commenced our analysis of mouse colon samples at 5 weeks of treatment. At this stage several ACFs and sporadic small broad based adenomas were usually detectable in the sensitive mouse strain A/J (Papanikolaou et al., 2000; Boivin et al., 2003).

Survivin expression was induced by DMH treatment as shown by Western blot analysis of the whole colon at 5 wks. After treatment (Fig. 1A) the Western blot clearly showed higher Survivin expression in the animals treated versus control animals (injected with vehicle solution).

The survivin expression pattern was depicted by immunohistochemistry at 5 weeks after treatment in the normal-appearing mucosa (non-ACF mucosa) of controls and treated animals (Fig. 1B, C) respectively. Survivin expression in controls was mostly located in the basal third of the crypts where few layers of positive cells are detectable. The mid third was weakly positive, the luminal surface and the intercrypt-table, which is the surface between two crypts (de Santa Barbara et al., 2003) were negative.

Survivin expression in treated animals appears homogeneously distributed in a monolayer of cells along the basal, middle and luminal compartments of the crypts; notably also the luminal surface and the intercrypt-table, where terminally differentiated colonocytes reside, are clearly positive (Fig. 1C). The signal is partly cytoplasmic and partly nuclear with a 5:1 ratio.

This Survivin change of expression in response to the DMH treatment mirrored the cryptal proliferative uppershift as shown by the modification of the MCM-6 protein expression (Fig. 1D, E).

Table 1. Tristar Tecnology Group 69570004.

HUMAN COLON AND RECTAL CANCER MACRO ARRAY	
14 Adenocarcinomas	
Grading Stage:	4 G1
	3 G2
	7 G3
6 Rectal Cancers	
Grading Stage:	2 G2
	4 G3
HUMAN SAMPLES FROM PATHOLOGY DEPARTMENTS	
6 Tubular Adenomas	
19 Adenocarcinomas	
Grading Stage:	4 G1
	3 G2
	12 G3

Table 2. Chemicon Tissue Macro Array TMA2901.

NORMAL HUMAN TISSUES	
Adrenal	Ovary
Bladder	Pancreas
Bone Marrow	Parathyroid
Cerebellum	Pituitary
Cerebral Cortex	Prostate
Breast	Skin
Diaphragm	Spinal Cord
Large Intestine	Spleen
Eye	Stomach
Fallopean Tube	Thyroid
Heart	Ureter
Kidney	Uterus
Liver	
Lung	
Lymph Node	
Muscle	

Survivin expression pattern in human and mice dysplastic and non dysplastic colon mucosa

In our analysis we compared Survivin expression patterns in human and mice colon.

In normal human colon mucosa dissected a few centimeters away the tumor formation, Survivin clearly showed a pattern of expression similar to the untreated A/J mice (Fig. 2, A and A'). A specific signal was mostly restricted to the basal compartment of the crypt, while in the middle compartment only a few cells were positive; the luminal compartment was negative.

Similarities between hTPM and DMH treated mice were also evident (Fig. 2B and B'): Survivin signal in both samples was much higher in all compartments of the crypts, lower, middle and upper third in the luminal portions (arrowheads), where terminally differentiated enterocytes are located, and in the intercrypt-table

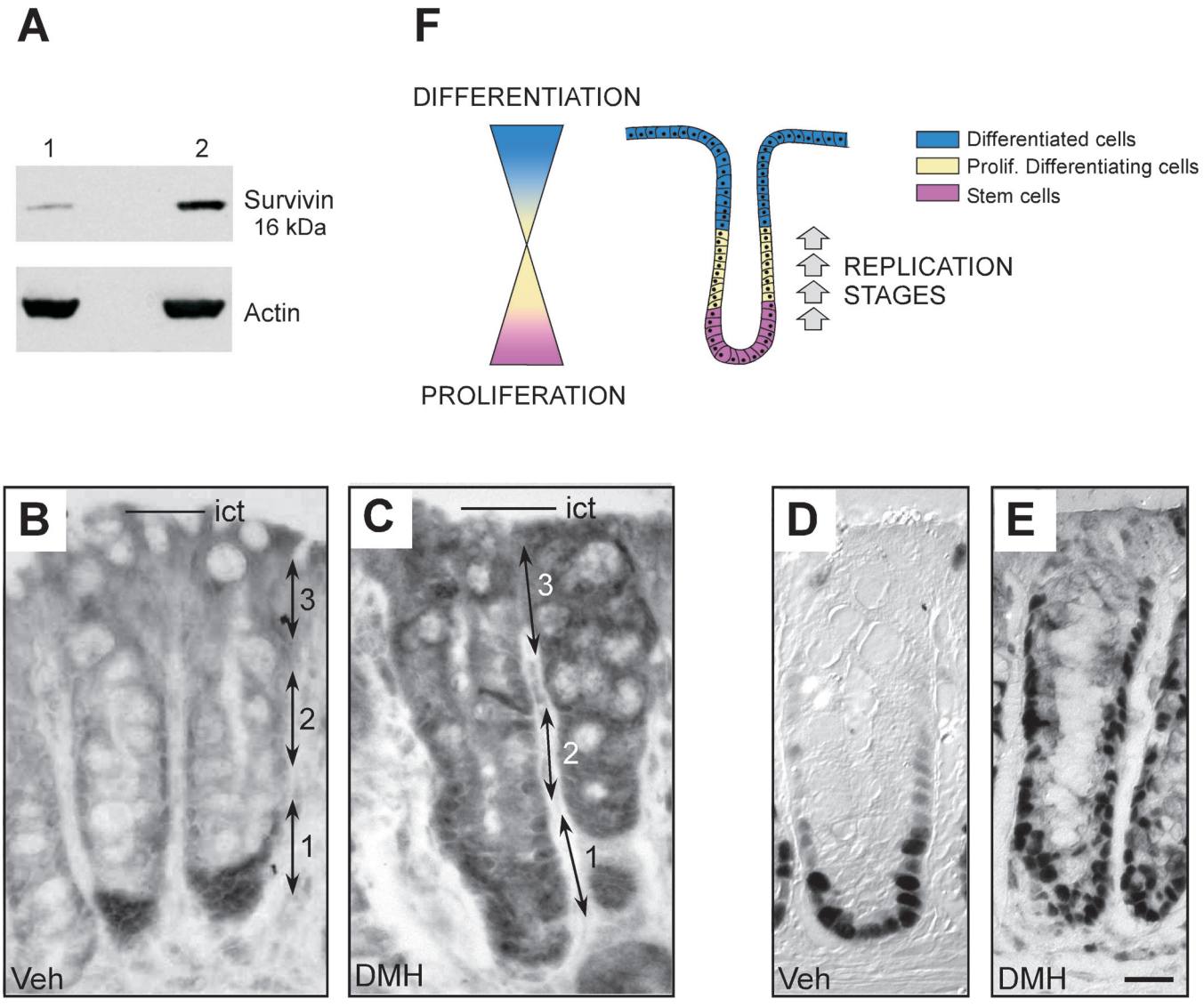

Fig. 1. Survivin expression in vehicle (1) and DMH treated mice (2) studied by Western blotting **(A)**: the intensity of the band is greatly increased in the DMH samples compared to the VEH treated control. ß-actin expression as loading reference (Actin). Survivin pattern in the colon of VEH treated **(B)** or DMH treated **(C)** A/J mice: in **B** vehicle treated colon crypts show Survivin expression restricted to the bottom of the crypts (1); in **C** Survivin is detected in DMH treated crypts, where the pattern of expression is greatly expanded in all the segments of the crypts (basal third (1), middle third (2), luminal third (3) and in the intercryptal-table (ict)). MCM-6 expression is restricted to the bottom of the crypt in VEH animals **(D)** while is present in all the compartements in DMH samples **(E)** (magnification bar: 40 μm). In **F** is showed a schematic drawing of the different areas of proliferation and differentiation of a colon crypt.

(arrows).

Survivin expression pattern in mice pre-neoplastic and neoplastic colonic lesions

Mouse samples collected at the above mentioned time points were analyzed. At a later stage after DMH induction, a grater number of displastic, pre-neoplastic and neoplastic formations were detectable in the colon affected mucosa.

Survivin specific staining was observed in the ACFs of DMH treated A/J mice. Survivin was present in all the compartments of the aberrant crypts but sometime the signal showed a non-homogeneous distribution; this observation is more commonly found in the displastic ACFs (Fig. 3A), while in the non displastic ACF, the signal is mostly equally distributed along the crypt (Fig. 3B).

In the tumors observed at 9, 15, 26 and 32 weeks, Survivin showed a more variable pattern of expression according to the histopathological characteristics of the tumor. In well differentiated broad based adenomas the signal was equally distributed in the adenomatous epithelium in the different cryptal compartments (basal, middle, luminal) (Fig. 3C). In adenomatous polyps from 9, 15, 26 weeks, Survivin expression pattern was more heterogeneous, with completely negative crypts detected in close proximity to intensely stained ones (Fig. 3D). In rectal adenocarcinomas dissected from mice at 15-26 and 32 weeks, the signal was similar to that described for adenomatous polyps: uniformly stained neoplastic crypts located next to completely negative ones. In some cases we observed an alternate Survivin specific signal distributed within the same transformed crypt, where adjacent clusters of Survivin positive and negative transformed epithelial cells were depicted (Fig. 3E, F', G'). Large areas of poorly stained transformed epithelial cells were more easily visible in poorly differentiated adenocarcinomas (Fig. 3E): in contrast, well and moderately differentiated adenocarcinomas were wider

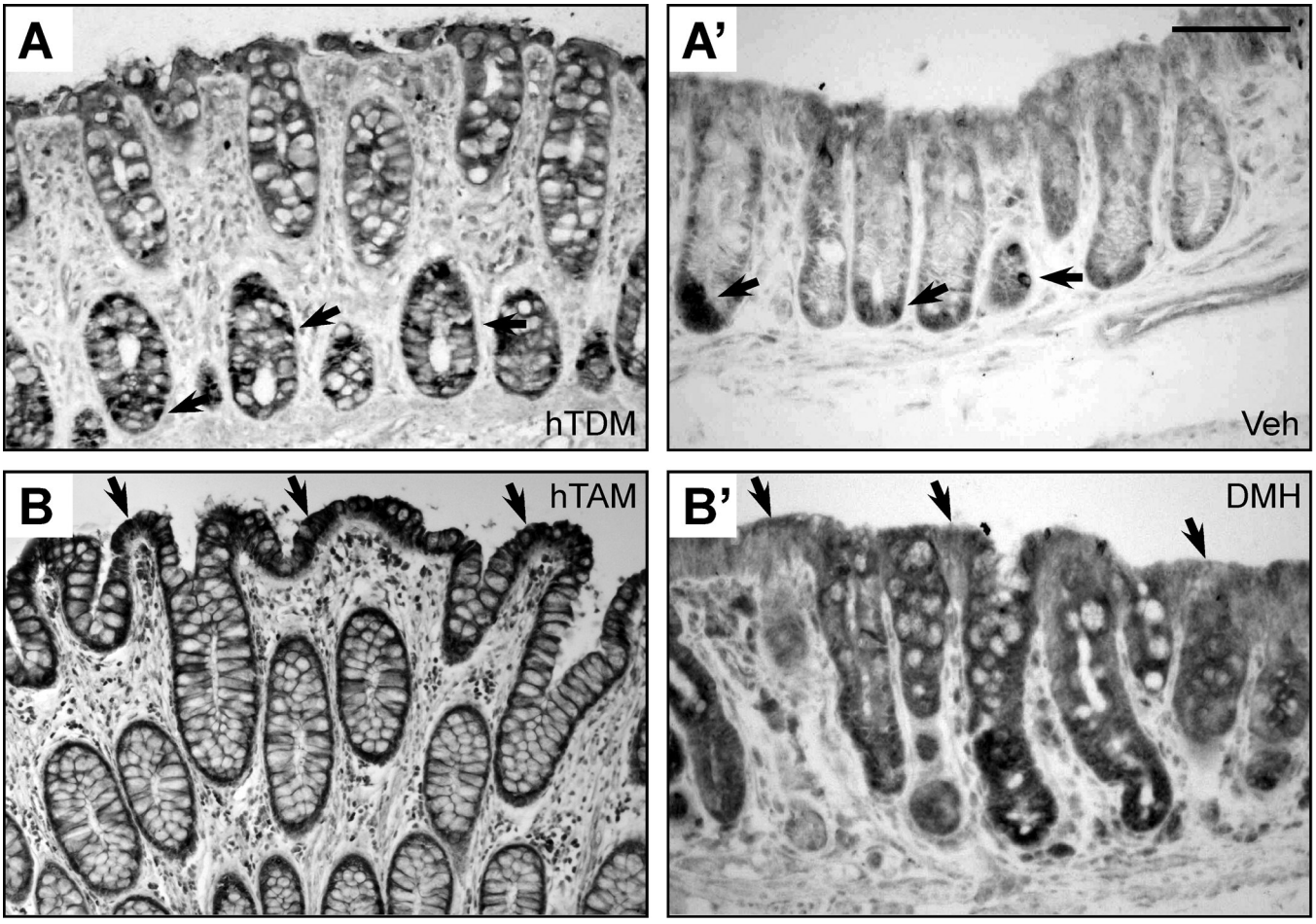

Fig. 2. Immunohistochemical staining for Survivin in human **(A; B)** and mouse samples **(A'; B')**, with **(B, B')** or without **(A, A')** DMH treatment. Bar: 120 μm.

and more intensely stained (not shown).

This suggests an inverse correlation between the displastic severity of the lesions and Survivin expression levels: the more severe the epithelial displasia, the less Survivin is expressed.

COX-2, ß-Catenin and Survivin expression patterns in human colon adenocarcinoma and in A/J mice in normal appearing non dysplastic (non-ACF) colon mucosa

In order to validate the possible role played by

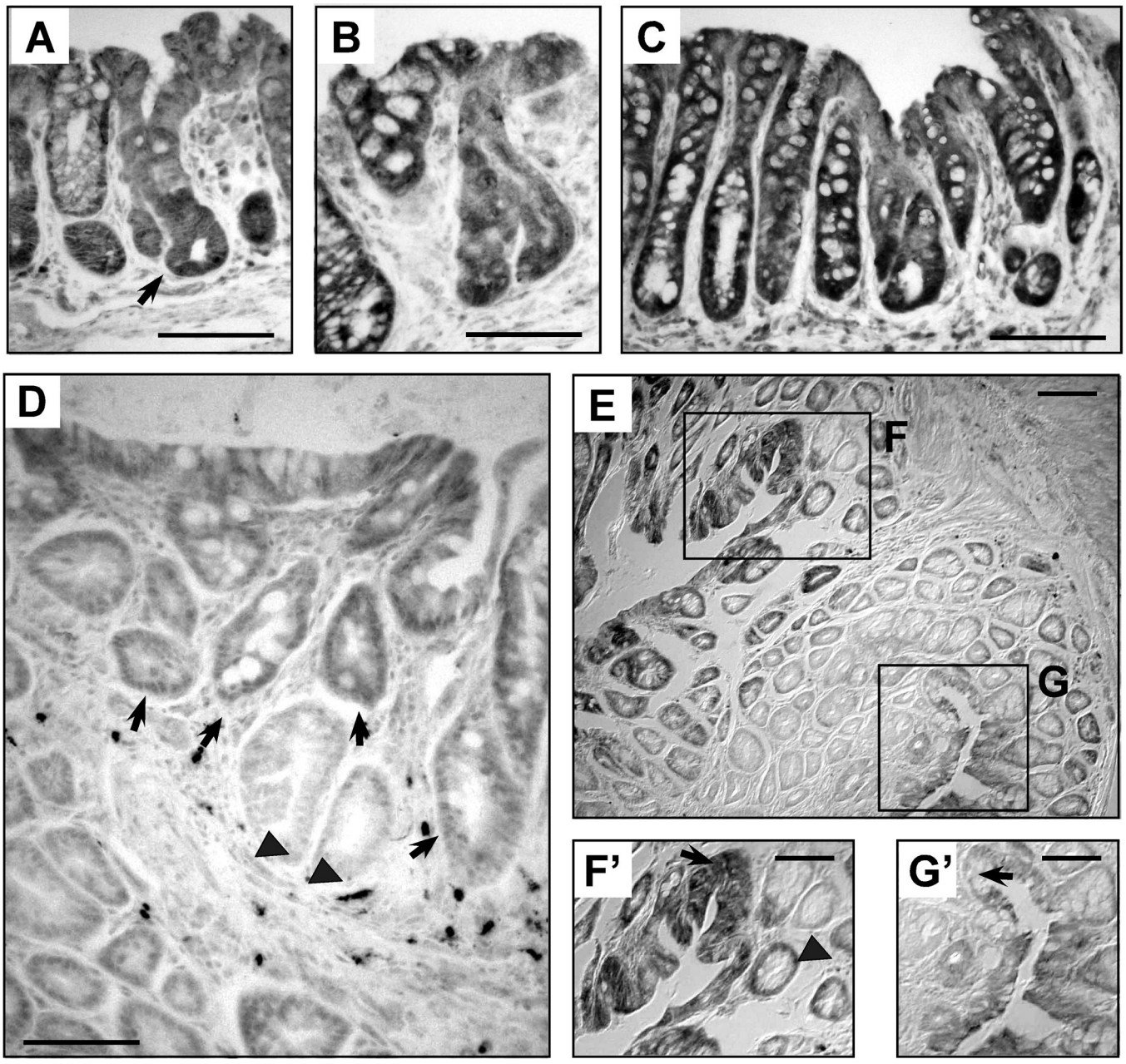

Fig. 3. Immunohistochemical staining for Survivin in mouse colon samples at different time point after DMH treatment: 9 weeks (**A**, displastic ACF; **B** non displastic ACF); 15 weeks (**C**); 26 weeks (**D**); 32 weeks (**E**). **F' and G'**, correspond magnification bar to boxed **F and G** areas shown in **E**. In **F'** crypts only partly positive are shown (arrow) in proximity to negative ones (arrowheads). This alternate pattern of expression is detectable also in the epithelium of the same crypts where areas of stained cells are shown in proximity of negative areas as shown in **G'** (arrow). Bars: A, C, D, E 120 μm; B, F', G' 30 μm.

COX-2 in modifying intracellular Survivin moieties, as suggested by *in vitro* studies (Li and Altieri, 1999; Kim et al., 2003; Krysan et al., 2004), we initiated a co-localization study of ß-Catenin, COX-2 and Survivin expression using adjacent sections from human and mouse colon cancer samples.

In vehicle treated mice COX-2 and Survivin showed a similar pattern of expression, both proteins being mainly localized in the basal third of the crypt. ß-Catenin staining was clearly restricted to the membrane compartment (no cytoplasmic and/or nuclear localization was observed). COX-2 specific signal was always restricted to the cytoplasm and the peri-nuclear area (Fig. 4).

To establish precisely if the COX-2 and Survivin positive cells were part of the proliferative region of the crypt, we ran an immunostaining for proliferation markers. In normal tissues proliferation markers such as

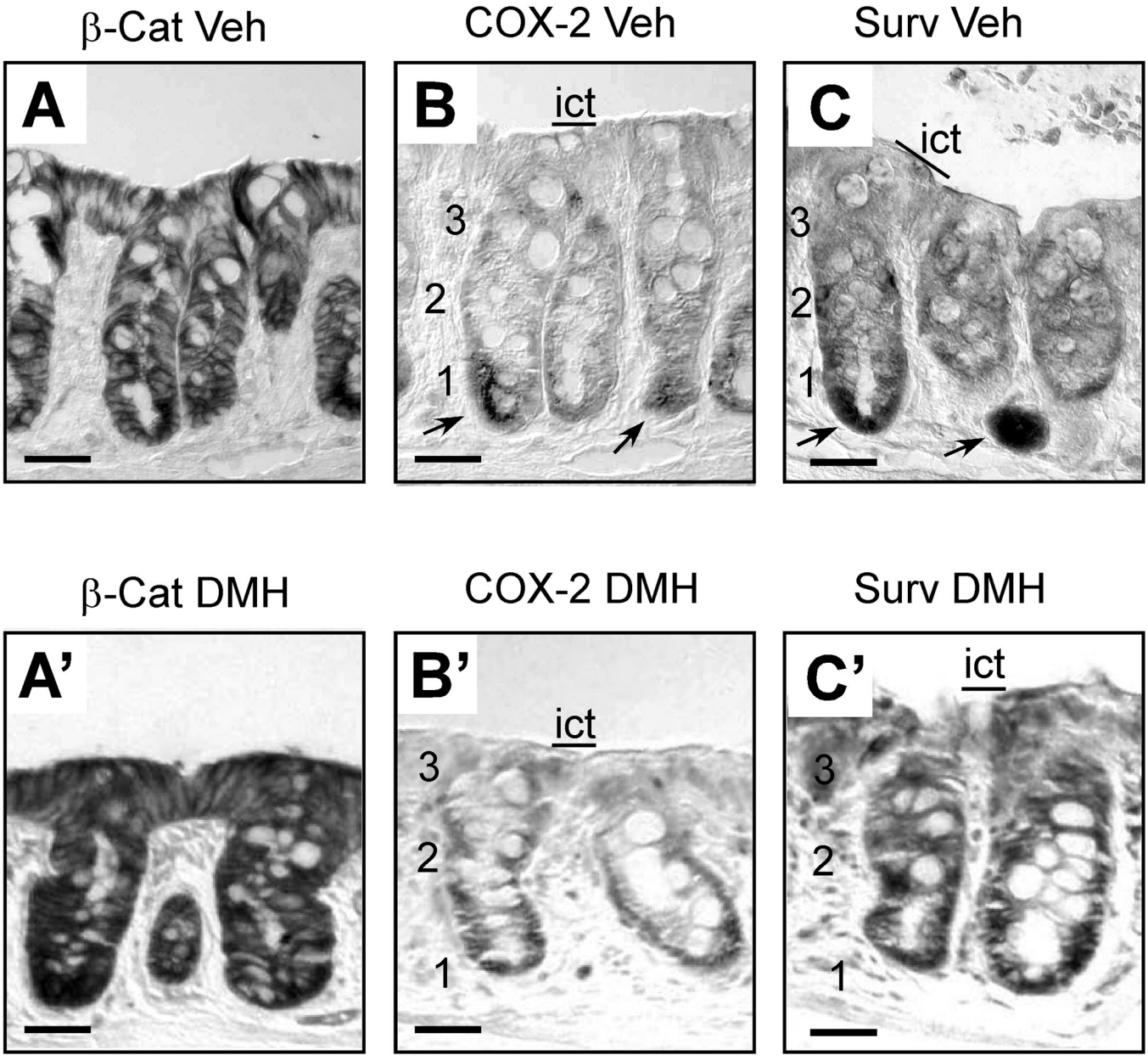

Fig. 4. Immunohistochemical staining for ß-Catenin, COX-2 and Survivin in vehicle (**A, B and C** respectively) and DMH treated mice (**A', B' and C'** respectively). Numbers identify the different compartment of crypt: basal third (1), middle third (2), luminal third (3). Ict stands for intercriptal table. Bar: 25 μm.

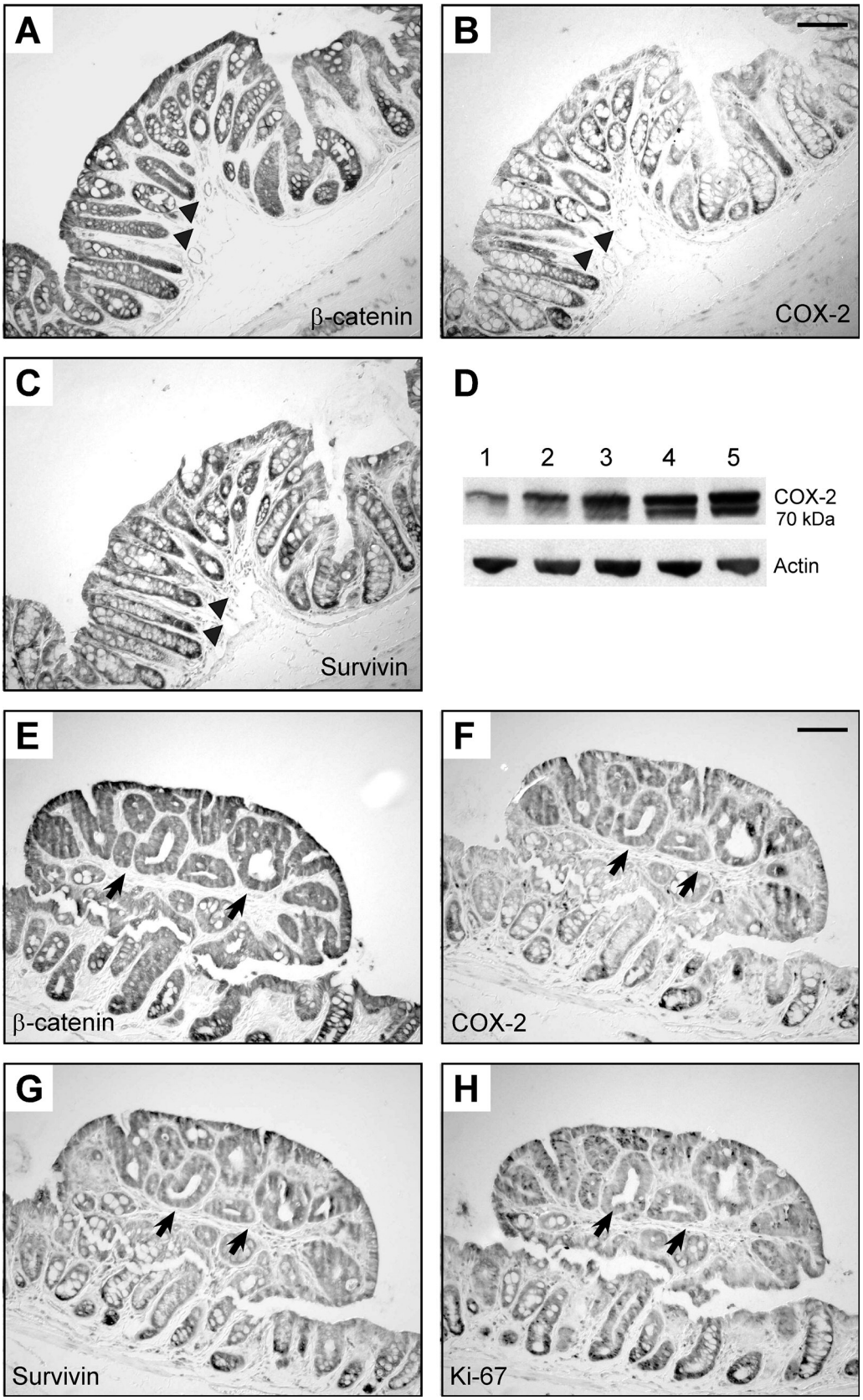

Fig. 5. Immuno-histochemical staining for ß-Catenin (**A, E**), COX-2 (**B, F**), Survivin (**C, G**) and Ki-67 (**H**). **A, B and C** show hyperplastic mucosa; **E, F, G, H** show a broad based adenoma. Western blot analysis for COX-2 at different induction stages is shown in **D** (Actin as a loading reference): vehicle sample (1) is compared to whole colon at 5 and 15 weeks (2, 3) and dissected tumors at 15 and 20 weeks (4, 5). Bar: 120 μm.

Ki-67 and MCM-6 (minichromosome maintenance protein-6) were restricted to the cells in the basal third of the crypts, where cycling stem cells normally reside (not shown).

After treatment with DMH, dramatic changes in the expression patterns of ß-Catenin, COX-2 and Survivin in the non-dysplastic (normal appearing) mucosa were revealed: ß-Catenin staining appears to be increased and is present also in the cytoplasm, COX-2 and Survivin were highly expressed not only in the basal third of the crypts, but also in the middle third. It is important to note that in the luminal surface and in the intercryptal-table they were expressed in terminally differentiated colonocytes (Fig.4 A',B',C'). Ki-67 and MCM-6 were no longer restricted to the basal third of the crypts but were detectable also in the middle and upper third of the crypts (not shown).

COX-2, ß-Catenin and Survivin expression pattern in human and in A/J mice ACFs dysplastic colon mucosa

The cellular distribution of ß-Catenin reflects its function. Localization of ß-Catenin primarily to the apical-lateral membrane signifies its role in cell adhesion, whereas cytoplasmic diffusion and/or nuclear migration suggest transcriptional activation of target genes. In this respect there is general consensus on the correlation between mutational status of cell lines, degree of de-differentiation and increase in free ß-Catenin (non cytoskeletal membrane bound) (Stewart and Nelson, 1997). Furthermore, an inverse correlation was established between the decreasing membranous and increasing nuclear detection of ß-Catenin in adenomas and carcinomas (Hao et al., 1997; Sheng et al., 1998).

COX-2 is consistently expressed in the tumor epithelium of more than 80% of human colorectal carcinomas and *in vivo* experiments have shown that COX-2 promotes angiogenesis (Masferrer et al., 1999) tumor cell invasion, and resistance to apoptosis (Gupta and Dubois, 2001). The pattern modifications of ß-Catenin, COX-2 and Survivin are clearly depicted in Fig. 5A-C respectively, in the hyperplastic colon mucosa at 15 weeks. Catenin expression was increased with a cytoplasmic and nuclear localization.

Survivin and COX-2 displayed homogeneous labeling in all the cryptal segments (lower, mid, and upper third) the luminal surface and the intercryptal-table. High expression was detected especially in areas where ACFs were developing.

COX-2 expression was higher in DMH treated colon samples at different induction end points (5-15-20 weeks after treatment) and in dissected tumoral masses at 15 and 20 weeks after treatment, as shown by Western blot analysis (Fig. 5D).

COX-2, ß-Catenin, Survivin and Ki-67 expression in

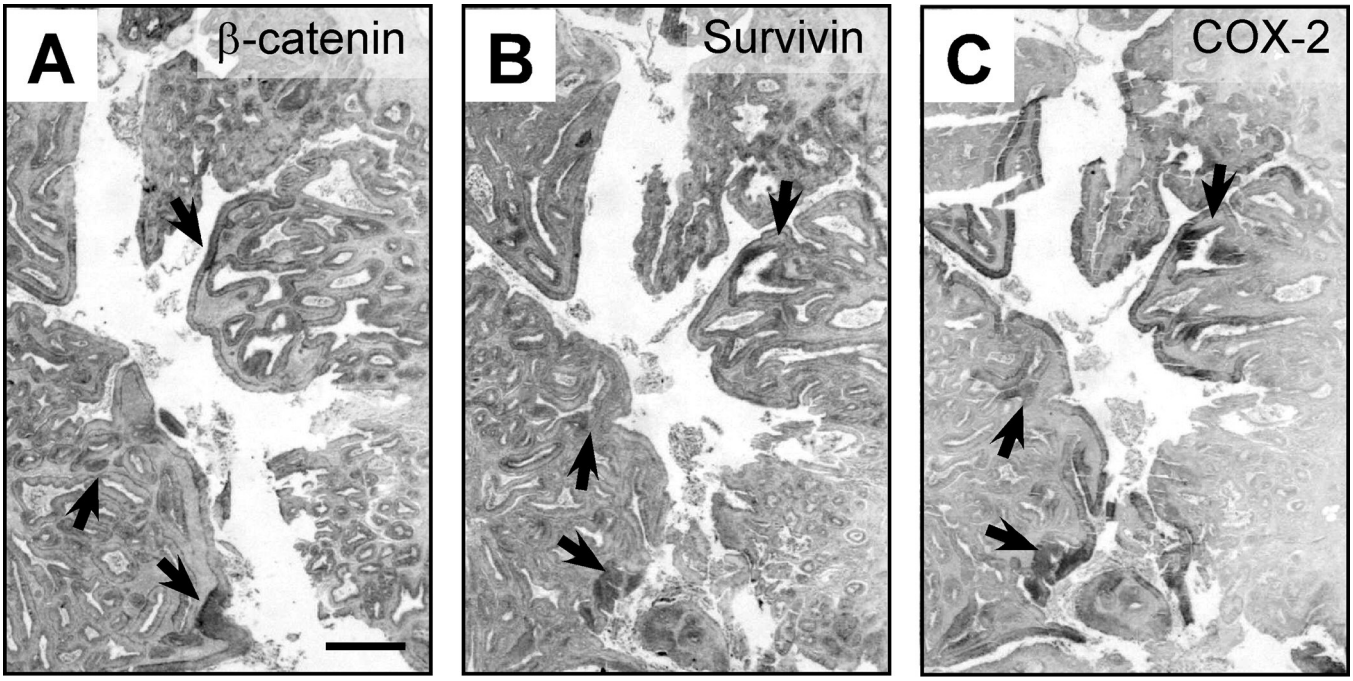

Fig. 6. Immunohistochemical staining for ß-Catenin (**A**), Survivin (**B**) and COX-2 (**C**). In human tubular adenomas ß-Catenin expression pattern is partly membrane bound and partly cytoplasmic; areas of sporadic increase in the cytoplasmic accumulation of the signal are depicted (**A**, arrows). Restricted clusters of Survivin positive epithelial cells (**B**, arrows) are detected in proximity of negative areas in the same crypts. Cox-2 signal parallels Survivin signal and ß-Catenin cytoplasmic signal in the same cells (**C**, arrows). Bars: 120 μm.

adenomatous polyps is shown in Fig. 5E-H respectively. Serial sections of a broad-based well differentiated tubular Adenoma are depicted, which still at 15 weeks was the most common neoplasm.

The adenomatous branching epithelial tubules showed labeling specific for COX-2. They depicted a

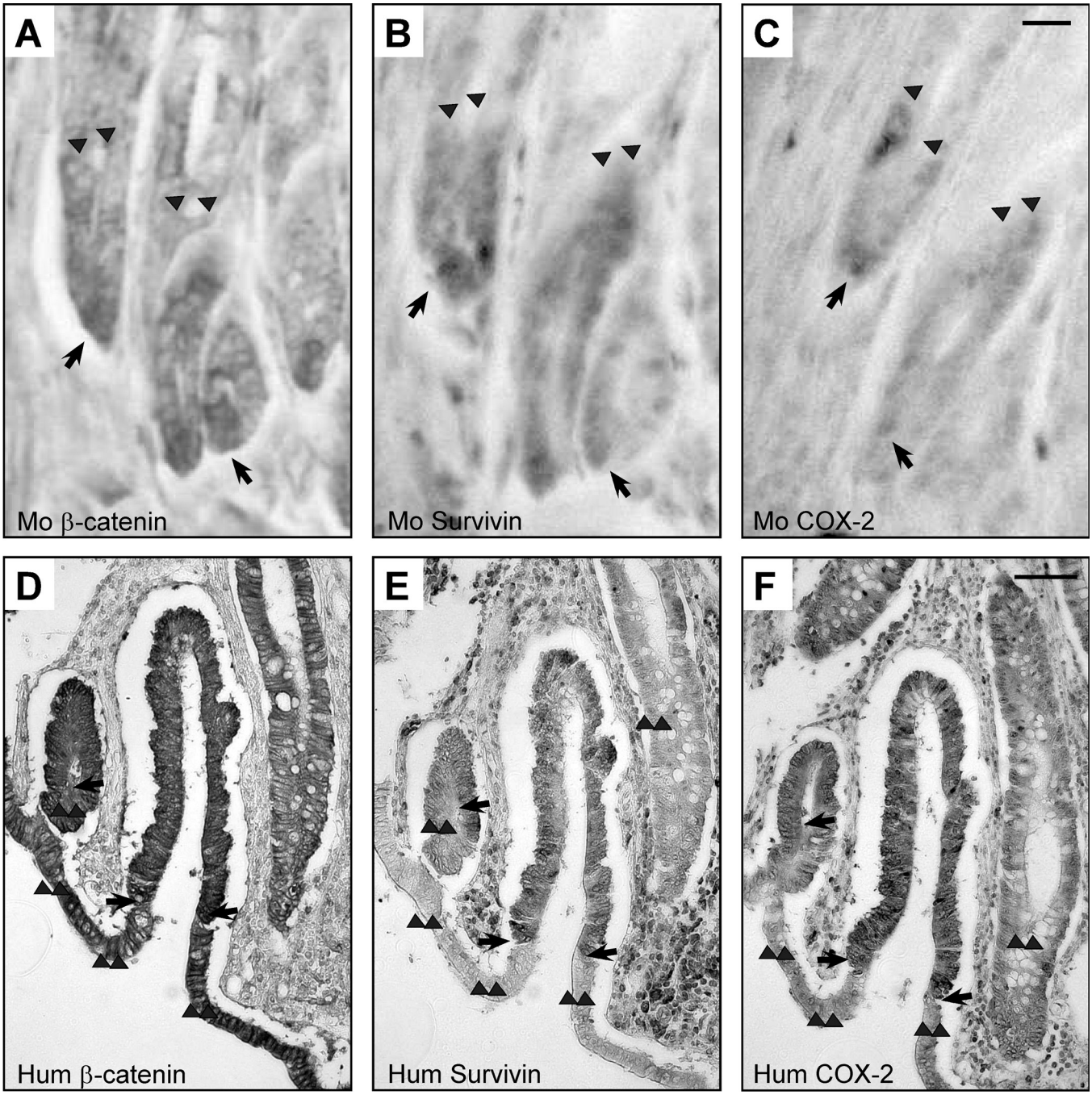

Fig. 7. Immuno-histochemical staining for ß-Catenin, Survivin and COX-2 in mouse invasive adenocarcinoma (**A, B, and C** respectively, 30 weeks of induction) and human adenocarcinoma (**D, E and F** respectively). Areas of sporadic increase in ß-Catenin accumulation are depicted (arrows); the increase of ß-Catenin expression is distributed in areas of the same crypt and starts with a sharp boundary (arrowheads). Restricted clusters of Survivin positive epithelial cells (arrows) are detected in proximity of negative areas in the same crypts; note the sharp boundary (arrowheads). Mouse and human patterns are comparable. Bars: 30 μm.

clear diffuse co-expression of Survivin and COX-2, while ß-Catenin showed a more diffuse pattern with an increased signal in the cytoplasm and nucleus, and a lower one in the membrane region.

Ki-67 and MCM-6 expression pattern clearly indicated an enhancement of proliferation (Fig. 5H).

In Fig. 6A-C respectively, ß-Catenin, Survivin and COX-2 expression patterns are shown in adjacent sections of human tubular adenomas (severe dysplasia). In Fig. 6A ß-Catenin pattern was partly membrane bound and partly cytoplasmic, with maximal reactivity in the goblet cells and nuclear reactivity almost absent. Survivin showed an alternate pattern of expression where, in the same crypt, positive clusters of epithelial cells were distributed next to negative ones (Fig. 6B). Fig. 6C showed that Cox-2 was exclusively expressed in Survivin positive epithelial cells.

Whilst in the adjacent negative epithelial cells ß-Catenin specific signal was restricted to the membrane compartment, in the areas where COX-2 and Survivin are co-expressed, the cytoplasmic expression pattern of ß-Catenin was more diffuse.

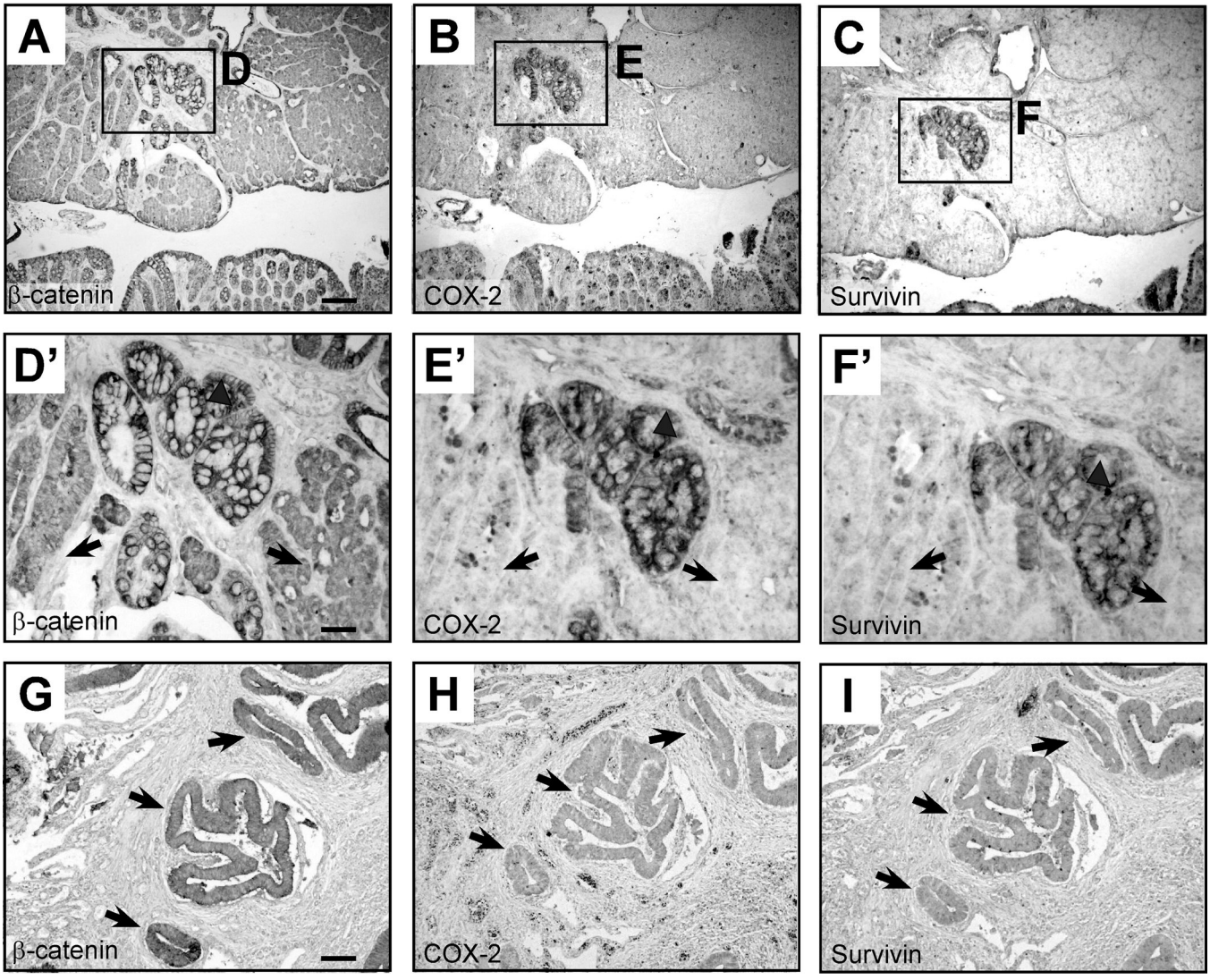

Fig. 8. Immunohistochemical staining for ß-Catenin (**A, D', G**), COX-2 (**B, E', H**) and Survivin (**C, F', I**) in 35 weeks mouse large invasive adenocarcinoma (**A-F'**) and human adenocarcinoma (**G-I**). COX-2 (**B**) and Survivin (**C**) co-expression in mouse is restricted to the square delimited areas (**D, E, F** showed at higher magnification in **D', E', F'**) which correspond to crypts with a membrane bound ß-Catenin pattern (arrowheads). In the majority of the surrounding tumoral mass where ß-Catenin has a more diffuse cytoplasmic and nuclear expression pattern, COX-2 and Survivin are not expressed (arrows). COX-2 (**H**) and Survivin (**I**) in human are co-expressed in the mutated crypts (arrows) where ß-Catenin (**G**) has a diffuse cytoplasmic and nuclear pattern of expression (arrows). Bar: A-C, 150 μm; D'-F', 50 μm; G-I, 45 μm.

COX-2, ß-Catenin and survivin expression pattern in human and mouse colon adenocarcinoma

Tissue Micro Arrays containing human colon cancer samples of different grading (G1, G2, G3) and nineteen human colon adenocarcinomas embedded in paraffin (Grading G1, G2, G3) (with the matching normal mucosa of a subset of them) (Table 1) were used in immunohistochemical experiments. The data obtained were compared to the expression patterns found in mouse samples.

A slight increase in immunoreactivity for COX-2 and ß-Catenin was observed in the adenomas compared to the neighboring mucosa hTPM. In the adenomas and in the hTAM COX-2 (not shown) and Survivin were co-expressed in all the segments of the crypts, lower, middle and upper third, in the luminal portions and in the intercrypt-table where terminally differentiated cells are located (not shown).

In the hTDM COX-2 and Survivin were restricted to the lower third of the crypts (crypt bottom) where proliferating stem cells are distributed. The immunoreactivity for both proteins was extremely low, close to the limit of detection (not shown). This pattern is clearly identical to the one already described for the vehicle and DMH treated mice colon mucosa (Fig. 2). In all the samples analyzed, Survivin and COX-2 co-expression was always restricted to colonic epithelial cells, in stromal cells such as fibroblasts, mononuclear inflammatory cells, endothelial cells and smooth muscle cells a COX-2 specific signal was detected mainly in the cytoplasm and in the perinuclear area (not shown).

In the hTDM, ß-Catenin immunoreactivity was exclusively distributed in the plasma membranes without any nuclear staining (data not shown). Areas of diffuse nuclear ß-Catenin specific immunoreactivity were observed in all the adenocarcinomas, while in the hTPM the pattern was mainly cytoplasmic (not shown). In Fig. 7 ß-Catenin, Survivin and COX-2 expression patterns are shown in adjacent sections of a mouse colon adenocarcinoma. COX-2 and Survivin co-expression were restricted to specific segments of three different crypts: the two expression patterns were extremely coincident in the same clusters of epithelial cells. In human adenocarcinomas several crypts showed an alternate pattern of expression, where Survivin-positive clusters of epithelial cells (arrows) were distributed next to negative epithelial cells (arrowheads) from the same crypt (Fig. 7B,C,E,F). As clearly shown in adjacent sections, COX-2 was exclusively expressed in Survivin-positive epithelial cells (arrows). The Survivin-negative epithelial cells (arrowheads) were also negative for COX-2 expression.

In the areas where COX-2 and Survivin are co-expressed, the cytoplasmic expression pattern of ß-Catenin was more diffuse, as shown in (Fig. 7A,D) (arrows). In the adjacent negative epithelial cells (arrowheads) ß-Catenin specific signal was more restricted to the membrane compartment.

In very large invasive adeno-carcinomas (late stages of induction) ß-Catenin showed a heterogeneous expression pattern with alternate areas of cytoplasmic, nuclear and membrane-bound localization in the same tumor mass (Fig. 8). In some of these invasive adeno-carcinomas Survivin and COX-2 were restricted to crypts where ß-Catenin is still mainly in a membrane-bound conformation (Fig. 8). In the areas where ß-Catenin has a more diffuse cytoplasmic and/or nuclear localization Survivin and COX-2 were not expressed (Fig. 8D,E,F). By contrast, in human samples we observed Survivin and COX-2 co-expression in poorly differentiated adeno-carcinomas where ß-Catenin had a cytoplasmyc and nuclear localization (Fig. 8G-I).

Survivin expression in normal human tissue

The analysis of the Tissue Micro Arrays revealed Survivin expression in several normal human tissues such as bone marrow, bladder, large intestine, fallopian tube, ovary, stomach and uterus (not shown). The signal was mainly nuclear in the bone marrow, in the large intestine, and in the proliferative portion of the intestine crypts (not shown); similar pattern was observed in the stomach glands, in the ovary, uterus and fallopian tube (not shown).

Discussion

The aim of this study was to investigate the expression pattern of Survivin during induction and tumor progression in colonic enterocytes of a susceptible mice strain (A/J) induced by DMH treatment. We compared mouse and human colonic hyperplastic and neoplastic lesions and correlated Survivin distribution with the expression of COX-2 and/or ß-Catenin, molecules which potentially affect Survivin protein transcription and stabilization. We also compared Survivin distribution with proliferative markers MCM-6 and Ki-67.

This paper for the first time clearly describes COX-2 and Survivin co-expression during CRC tumor progression, as well as an up-regulation of Survivin expression in A/J mice treated with DMH.

Survivin expression pattern in humans and mouse

The main function of the normal colon epithelium is to reabsorb water and, in a simplified view, it is composed of two differentiated cell types: the enterocytes (or colonocytes) and the goblet cells (Karam, 1999). Goblet cells are in prevalence distributed in the mid-crypt and are characterized by mucus vacuoles, while enterocytes are the absorptive cell type and are mainly located at the top of the crypt and in the intercrypt table. A third class of enteroendocrine cells is mainly at the base of the crypt (de Santa Barbara et al., 2003). Stem cells reside at the bottom of the crypt, which is characterized by proliferation (Potten et al., 1992), and some of them differentiate into transit cells that continue their migration and proliferation in the

mid-third of the crypt, giving rise to terminally differentiated cells (Bach et al., 2000; Brittan and Wright, 2002). Terminally differentiated cells (the majority of which are enterocytes) reside in the upper third of the crypt in the luminal portions and in the intercryptal table (Karam, 1999; de Santa Barbara et al., 2003). At this level physiological apoptosis takes place.

According to our observations in the apparently normal colon mucosa (no ACF mucosa), after 5 weeks of DMH exposure there was an increase in Survivin expression and a topological change of its localization pattern compared to the control colon mucosa: these data are also comparable with similar staining performed on hTPM. Survivin is no longer restricted to the basal compartment of the crypt, but starts to be detected in all segments and in terminally differentiated enterocytes of the luminal third and intercryptal table (Karam, 1999; de Santa Barbara et al., 2003).

This new pattern of expression detected in the apparently normal colon mucosa and in the aberrant crypt *foci* coincides with the upward expansion of the proliferative cryptal compartment as we have shown by Ki-67 and MCM-6 specific immunostaining.

In the last few years, elements of the mini-chromosome maintenance (MCM) family have been studied in cancer. MCMs are involved in the initiation of DNA replication and are crucial in controlling that this phenomenon occurs only once during the cell cycle (Kearsey and Labib, 1998; Labib et al., 2000). These proteins show upregulated expression levels in tumor cells and some elements are much better indicators of a wide variety of cancer than traditional biomarkers (Semple and Duncker, 2004) Heidebrecht et al., 2001 have performed an interesting comparison of the distribution profile of MCM-6 and the Ki-67 antigen, showing that the former has the characteristics required to be a prognostic marker in cancer management. Similar studies have been performed on other elements of this family, e.g. MCM-7 (Padmanabhan et al., 2004), showing that MCM elements are better discriminatory markers of proliferation between benign epithelium and invasive adenocarcinoma than Ki-67. For all these reasons the comparison of Survivin and MCM-6 expression is crucial in monitoring the first steps of cancerogenesis.

DMH treatment induces an upward-shift of the proliferation zone extended from the base to the luminal third of the crypt, changing the topography of proliferating and apoptotic cells; in fact within each crypt one-third of the proliferating activity is distributed in the middle compartment, and roughly 3% in the luminal third (Kozoni et al., 2000). In contrast, after DMH treatment apoptosis is almost all concentrated in the basal compartment of the crypt (Kozoni et al., 2000). Different studies on colon cancer samples from human patients and from DMH/AOM mouse model indicate a significant cyclin D1, cyclin E and PCNA expression increase in the apparently normal colon mucosa and in the aberrant crypt foci, adenomas and adenocarcinomas at both RNA and protein level (Shpitz et al., 1997; Hur et al., 2000; Khare et al., 2003). This upward extension of the proliferative compartment in colon rectal cancer has been consistently observed, suggesting that increased cell proliferation is a prerequisite for tumor progression both in humans and in the DMH/AOM mouse model (Shpitz et al., 1997; Kozoni et al., 2000; Wali et al., 2002).

The localization of Survivin in Ki-67 positive nuclei was also reported by Mahotka et al. (2002), therefore the changes in proliferation and apoptosis activity in both humans and mice could be also due to the upward-shift of Survivin expression pattern within the crypt, as a clear consequence of Survivin dual function as mitotic regulator and apoptosis inhibitor (Fortugno et al., 2002; Mahotka et al., 2002). Furthermore it is well known that Survivin increases Aurora B kinase activity, a protein which affects cell cycle regulation and chromosomal segregation (Bolton et al., 2002; Chen et al., 2003).

Any escape from apoptosis impairs the capability of metazoans to control the homeostasis and morphogenesis of normal tissues (Abrams, 2002; Johnstone et al., 2002). Any sort of interference that reduces the efficacy of this process, creating an abnormal increase in cellular life span, is considered a possible contributor to the accumulation of aberrant mutations which in turn favor carcinogenesis (Thompson, 1995). Our data indicate that Survivin can play a crucial role in the mechanisms responsible for DMH induced hyperproliferation and tumor progression in mice and Sporadic Colon Cancer in humans. In terminally differentiated colonocytes the high level of Survivin expression and the concomitant absence of apoptosis in colon cancer (Shiff and Rigas, 1997; Kozoni et al., 2000), lend credence to the possibly predominant anti-apoptotic and cell-cycle regulatory role of Survivin, as reported by Altznauer et al. (2004). In this regard it is important to note that COX-2 and Survivin expression patterns always coincided, even at early stages of tumorigenesis (5 weeks) in DMH treated animals.

At later stages Survivin and Cox-2 co-expression was heterogeneous in polyp adenomas and adenocarcinomas, showing an alternate pattern of expression with positive areas close to completely negative ones, a pattern mostly similar to the human samples of comparable stage that we analyzed.

The grading of the neoplastic lesions suggests a correlation between dysplasia appearance and increase in Survivin expression (Gianani et al., 2001).

In adenomas and adenocarcinomas, Survivin expression seems to correlate with the level of differentiation displayed by the tumor formations, although some discrepancies at late stages of tumorigenesis are present.

The decrease and/or loss of Survivin expression in the poorly differentiated cells of colonic tumors, although attributable to genomic instability in humans (Gianani et al., 2001) cannot be applied to our mice model of colon cancer, since genomic instability in this model, and more generally in rodents, is quite a rare

event (Guda et al., 2004). Although some Authors define Survivin as undetectable in human adult tissue, we confirm the detection of this protein in normal human tissues and particularly in the lower third of the colonic crypts as already described by Gianani et al and Zhang et al (Gianani et al., 2001; Zhang et al., 2001).

In normal human/mouse colonic mucosa, Survivin is expressed in restricted areas where proliferation is apparent and mitotic activity is the prominent cellular activity (Bach et al., 2000; Brittan and Wright, 2002).

COX-2, ß-Catenin and survivin expression

Current carcinogenesis models are based on the accumulation of genetic abnormalities, which progressively affect critical metabolic pathways essential for the homeostasis and morphogenesis of normal tissues (Kinzler and Vogelstein, 1997; Hanahan and Weinberg, 2000). Two important pathways in the pathogenesis of CRC have been determined: the Wnt signaling pathways and the metabolic conversion of arachidonic acic to prostaglandins (PGs) via the cyclooxygenase-2 (COX-2) (Prescott and Fitzpatrick, 2000; Nishihara et al., 2003).

As previously described, during CRC tumor progression, the Survivin pattern becomes more heterogeneous (Gianani et al., 2001) showing completely negative crypts detected in proximity of positive ones (adenomatous polyp and adenocarcinoma). This varying pattern of expression is more extremely manifested in adenocarcinomas and colon rectal carcinomas, but we also observed it in Adenomas. In human and mice samples we clearly observed for the first time that this alternating Survivin pattern restricted to the epithelium of the same single crypt coincides with an identical alternating COX-2 expression pattern. As a consequence, clusters of cells expressing both Survivin and COX-2 are observed adjacent to completely negative epithelial clusters in the same mutated crypt. We also showed that the Survivin/COX-2 clusters of positive epithelial cells often display a more diffuse cytoplasmic ß-Catenin pattern.

Our data clearly suggest that the modification of Survivin pattern during colon rectal cancer tumor progression not only mirrors the cryptal proliferative uppershift in the colon mucosa in hTPM and DMH treated mice, but also coincides with the expression of other potentially regulatory proteins during all stages of tumor progression. These proteins can affect both Survivin transcription, as suggested by in vivo data (Kim et al., 2003) and stabilization as indicated by studies from non-small cell lung cancer (Krysan et al., 2004). The Wnt signaling pathway APC gene (adenomatous polyposis coli) is the most frequently targeted gene for mutation in human CRC (Kinzler and Vogelstein, 1997; Kim et al., 2003; Clevers, 2004). In CRC, mutations of the APC gene lead to the aberrant formation of ß-Catenin/TCF4 complex which is the downstream effector of the Wnt signaling cascade. It is involved in the inappropriate up-regulation of several genes, which in turn may facilitate tumor progression (Korinek et al., 1997; Morin et al., 1997; Clevers, 2004). Recent evidence indicates that in human colorectal cancer Survivin can be transcriptionally up-regulated by the ß-Catenin/TCF complex, which imposes a stem cell mutated phenotype on several colonized colon crypts, coupling enhanced cell proliferation with resistance to apoptosis (Kim et al., 2003). Furthermore in DMH induced CRC tumor formation correlates with the expression of ß-Catenin-accumulated crypts (Hata et al., 2004).

Crypt stem cell survival in the mouse intestinal epithelium is regulated by Prostaglandins synthesized by COX-1, a constitutively expressed cyclo-oxygenase considered a housekeeping gene (Cohn et al., 1997), and by COX-2, which is a mitogen-inducible form of cyclo-oxygenase (Eberhart et al., 1994). COX-2 is up-regulated in human CRC (Eberhart et al., 1994) and related to tumor progression (Knosel et al., 2004). It is also over-expressed in (DMH/AOM) induced colon carcinogenesis in mice (Takahashi et al., 2000; Dong et al., 2003).

From our observations, Survivin/COX-2 co-expression during CRC progression suggests that COX-2 over-expression and the concomitant increase in PGE2 synthesis could possibly down regulate Survivin ubiquitation and its proteasomal degradation, stabilizing the protein and increasing its mitotic/anti-apoptotic activity, according to a mechanism observed in lung cancer (Krysan et al., 2004).

Taken together, the zonal ß-Catenin cytoplasmic diffusion and COX-2 over-expression can induce a general increase of cell proliferation and reduced sensitivity to apoptosis (Kotsinas et al., 2002; Iurlaro et al., 2004), possibly also through Survivin transcriptional up-regulation, and protein stabilization. This is also in agreement with the observation that in human colon cancer cell lines COX-2 is up-regulated by nuclear ß-Catenin accumulation and Ras pathways (Araki et al., 2003).

Finally, Survivin's function as a mitotic regulator and apoptosis inhibitor (Fortugno et al., 2002; Mahotka et al., 2002), will contribute to CRC tumor induction and progression.

These conclusions can be partly extended to both human and mice CRC with some further considerations. In contrast to human cells, mouse Survivin promoter is devoid of ß-Catenin/TCF4 binding sites (Kim et al., 2003; Iurlaro et al., 2004) and, although in the AOM/DMH rodent model APC it is rarely mutated, ß-Catenin mutations were frequently reported (Takahashi et al., 2000). This indicates that also in the AOM/DMH induced colon carcinogenesis there is an increase of oncogenic signaling from non E-cadherin bound ß-Catenin in hyperproliferating colonocytes, as observed in a well established mouse model of colon hyperproliferation/hyperplasia (TMCH) (Sellin et al., 2001).

Therefore, in the AOM/DMH mice model of colon carcinogenesis, Survivin over-expression could be partially modulated by COX-2/PGE2 induced protein

stabilization, as above mentioned, but apparently not by the transcriptional up-regulation mediated by ß-Catenin/TCF4.

This discrepancy in Survivin transcriptional regulation among humans and mice could partly explain the lack of Survivin and Cox-2 expression in large areas of mouse late invasive adeno-carcinomas (32 weeks) where ß-Catenin had a mainly cytoplasmic and nuclear localization. It is worth noting that in restricted compartments of the same tumors the crypts where there is a ß-Catenin membrane bound accumulation were the only ones to co-express Survivin and Cox-2. This restriction pattern can be considered a peculiarity of this murine system, at late stages of tumorigenesis.

In this respect we have to consider some other peculiarities of the AOM/DMH model. In particular p53 is almost never mutated (Nambiar et al., 2002; Aizu et al., 2003) but the colon-specific decrease in p300 expression due to the organ-specificity of the carcinogen has been recently reported (Aizu et al., 2003). p300 is a p53 transcriptional co-activator required to induce specific p53 DNA-binding and transcriptional activation of cell cycle arrest genes such as p21 (el-Deiry et al., 1993). p300 down regulation can thus impair at least in part p53 tumor suppression activity (Aizu et al., 2003). Since Survivin is a p53-repressed gene (Hoffman et al., 2002), this colon specific drop in p300 expression could play an important role in tumor induction and progression.

A similar phenomenon is also observed in human sporadic colon cancer, but in this case it is not p300 down regulation that determines the failure in p53 tumor suppression activity, but the number of direct mutations which easily affect p53 physiology in human patients (Berger et al., 2003).

Survivin aberrant expression in the tumor-neighboring mucosa of humans and mice suggests that Survivin-positive tumors may develop from a Survivin positive field of normal appearing mucosa, in accordance with the "Field defect" principle (Bernstein et al., 2000; Sarela et al., 2000). In fact this principle indicates that in the normal flat mucosa the advent of a "field" with reduced apoptosis capabilities predisposes to cancer development and progression. The advent of such a field depends also on Survivin stabilization induced by COX-2 co-expression in the same epithelial cells. This observation is in accordance with Ohomori et al (Ohmori et al., 1999) that describes an increase of Bcl-2 in the tumor neighboring colon mucosa.

Conclusions

In conclusion, we report for the first time Survivin over-expression and a COX-2 and Survivin pattern of co-expression during the early phases of CRC (colon rectal cancer) tumor induction, in the normally appearing colon mucosa and during all phases of tumor development up to the carcinoma stage in DMH treated A/J mice. We described and compared this pattern also in human colon adenoma and carcinoma clinical biopsies and at the corresponding tumor stages . In these samples the zonal co-expression of ß-Catenin/Cox-2/Survivin confirms *in vivo* the detection in the same cells of regulatory proteins either belonging to an oncogenic signaling pathway that specifically up-regulates Survivin transcription (ß-Catenin), or to a class of specific growth and/or mitogen-inducible form of cyclo-oxygenase COX-2, which activate the synthesis of metabolites which can stabilize Survivin intracellular moieties (PGE2).

Our data corroborate previous studies and lend credence to the hypothesis of a specific cooperative role played by ß-Catenin/COX-2 pathways, which can modulate the concentration and/or activation of Survivin and other key proteins during colon rectal cancer progression, even in restricted compartments of the same tumoral mass, as suggested by the alternating pattern of expression observed with some difference in humans and mice samples.

For the importance of zonal COX-2 and COX-2 inducing molecules expression in human colon rectal cancer see also Maihöfner et al. (2003).

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