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Immunolocalization of interleukin-1 receptor antagonist in healthy and infarcted myocardium

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Summary. Ischemic heart disease is a widespread cause of death. During infarction, myocardial injury is mediated by release of several pro-inflammatory cytokines including multifunctional interleukin-1 (IL-1). In various tissues, IL-1-mediated deleterious effects are known to be attenuated *via* the over-expression of natural anti-inflammatory cytokine IL-1 receptor antagonist (IL-1ra). In the present investigation, IL-1ra distribution in healthy and infarcted myocardium was studied by light and electron microscopy. After immunostaining, weak positivity resulted for cardiomyocytes in normal myocardium and, at higher degrees, in infarction border areas and ischemic ones. In ischemic areas, additional reactivity was displayed by the extracellular matrix and intravascular plasma. Immunogold labelling provided further details on intracytoplasmatic and extracellular distribution; in particular, noticeable gold particle distribution appeared on intercalated discs in normal and hypertrophic cardiomyocytes, as well as on thickened Z-lines for these latter. The present results suggest that cardiomyocytes represent a major source of IL-1ra *in vivo*, even though additional contribution by blood derived IL-1ra is to be taken in account in ischemic areas. In addition, ischemia-associated intracytoplasmic IL-1ra increase and its additional presence in the extracellular matrix is consistent with the concept that this cytokine plays a cardioprotective role at different levels and by distinct mechanisms.

Key words: Interleukin-1 receptor antagonist, IL-1ra, Cardiomyocyte, Myocardium infarction, Heart

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Introduction

Among the members of Interleukin family, interleukin-1 (IL-1) consists of two agonists, IL-1α and IL-1ß, and a specific receptor antagonist, IL-1ra, each acting as a ligand for two receptors termed IL-1R type I and IL-1R type II (Arend et al., 1998). The molecule now known as IL-1ra was originally referred as an IL-1 inhibitor in the supernatants of human monocytes cultured on adherent IgG (Arend et al., 1985). Then, this first isoform of IL-1ra was also reported to be secreted from macrophages, neutrophils, hepatocytes, endothelial cells and microglial cells and is now termed sIL-1ra (Arend et al., 1998). Besides the secreted isoform, three additional IL-1ra intracellular isoforms have been described to date. One of these, termed icIL-1ra1, was originally found in epithelial cells (Haskill et al., 1991) and subsequently in monocytes, macrophages, fibroblasts, and endothelial cells (Arend and Guthridge, 2000). A cDNA coding for a second intracellular isoform, termed icIL-1ra2, was cloned from human leukocytes (Muzio et al., 1995), even though this predicted protein was never found to be expressed *in vivo*.

The third isoform, termed icIL-1ra3, was mainly found to be expressed in neutrophils and hepatocytes (Malyak et al., 1998) and is also detectable in smaller amounts in monocytes, macrophages, and epithelial cells (Arend and Guthridge, 2000).

IL-1 isoforms are reported to play a key role in the complex cytokine network, regulating important functions during development, cell differentiation, tissue remodelling, infection, inflammation and cell death (Loppnow et al., 2001; Arend, 2002). On the other hand, IL-1ra detection in patients with inflammation, infection, allergic disease, cancer and various postoperative conditions is consistent with its involvement in mediating anti-inflammatory responses in a wide variety of anatomical districts such as joints (Firestein et al.,

1992; Arend and Gabay, 2000), gastrointestinal tract (Sekiyama et al., 1994; Casini-Raggi et al., 1995; Daig et al., 2000; Mayer et al., 2000), lung (Mikuniya et al., 2000; Hagaman et al., 2001), kidney (Pereira et al., 1994; Tam et al., 1994) and central nervous system (Loddick et al., 1997; Wong et al., 1997; Allan, 1998). Thus, IL-1ra seems competitively to block IL-1 agonistic isoforms binding to cell surface receptors, regulating their multifunctional effects in various tissues (Arend et al., 1998; Arend and Guthridge, 2000; Arend, 2002).

Since IL-1ra can be virtually expressed by any IL-1-producing cell, it is likely that correct balance between IL-1 and IL-1ra in local tissues is crucial for the maintenance of physiologic conditions, whereas imbalance between these cytokines can lead to pathological disorders (Arend and Guthridge, 2000; Arend, 2002). There are many evidences supporting the concept that perturbations of IL-1/IL-1ra ratio are involved in the pathogenesis of several cardiovascular tissue diseases (Loppnow et al., 2001). Particularly, elevated amounts of IL-1 isoforms have been detected in human atherosclerotic arterial wall (Tipping and Hancock, 1993; Galea et al., 1996) and in hearts from patients affected by dilated cardiomyopathy (Francis et al., 1998; Ukimura et al., 2003) and ischemic disease (Oyama et al., 2001; Pomerantz et al., 2001).

Elevated levels of IL-1 isoforms were also detected in sera from patients affected by myocarditis (Matsumori et al., 1994; Abe et al., 2004) or myocardial infarction (Guillen et al., 1995; Balbay et al., 2001).

Further, it has been reported that cardiomyocytes can produce IL-1 *in vitro* in response to injury (Müller-Werdan et al., 1998). Likewise, IL-1ra was reported to be involved in atherosclerosis (Dewberry et al., 2000), chronic congestive heart failure (Missov et al., 1997), and myocarditis (Abe et al., 2004). Moreover, elevated IL-1ra serum levels were found in patients with acute myocardial infarction (Latini et al., 1994; Shibata et al., 1997; Patti et al., 2004). However, it remains unclear which is/are actual source/s for IL-1ra in human infarcted myocardium.

Several studies evidenced that apoptosis is involved in the pathogenesis of various cardiovascular diseases (Saraste et al., 1997; Yaoita et al., 2000; Akasaka et al., 2006). Moreover, possible correlation between apoptosis and IL-1 family cytokines has been reported. Particularly, animal models of ischemia-reperfusion (I/R) injury were used to demonstrate that induced over-expression of IL-1ra (Suzuki et al., 2001) or selective inhibition of IL-1 (Yaoita et al., 1998) caused a reduction of infarct size, inflammatory response and IL-1-mediated apoptosis, preventing ischemia-induced myocardial dysfunction.

The present study focuses on the expression of IL-1ra in human healty and infarcted myocardium and the obtained results strongly suggest that the major source of IL-1ra *in vivo* is represented by cardiomyocytes supporting the concept that this cytokine plays a cardioprotective role at different levels and by distinct mechanisms.

Material and methods

Sampling

Human myocardium samples were excised from (i) nonischemic areas, (ii) border areas, and (iii) ischemic areas of eight hearts explanted from cardiac transplant recipients secondary to myocardial infarction (n=8; 6 males and 2 females; mean age was 52 years). Additional samples were excised from two autoptic hearts from noninfarcted patients (n=2; 1 male and 1 female; mean age was 56 years).

After fixation with 4% neutral buffered paraformaldehyde, the samples were reduced and grouped into lot 1, for light microscopy processing, and lot 2, for electron microscopy processing. Samples of lot 1 were routinely dehydrated and embedded in paraffin. Serial sections were cut at 5µm thickness and mounted on poly-L-lysine precoated slides. Histological sections were stained with picro-Sirius red and hematoxylin or subjected to immunohistochemical reactions. Samples of lot 2 were dehydrated in graded ethanols and embedded in LR-White resin. Semithin sections were stained with 1% toluidine blue in aqueous solution. Ultrathin sections were collected on nickel grids and subjected to immunogold labelling reactions.

Immunohistochemical detection of IL-1ra

Deparaffinized and rehydrated histological sections from lot 1 samples were incubated for 10 min in 0.1% Triton X-100 solution to improve membrane permeability; endogenous peroxidase activity was inhibited by 5 min incubation in 3% H_2O_2; masking of non-specific binding sites was performed by incubation in 3% normal donkey serum for 30 min. Subsequently, sections were incubated with goat anti-human 1:25 diluted IL-1ra polyclonal antibody (Santa Cruz Biotech. Inc., CA, USA), for 2h, and finally with peroxidase-conjugated donkey anti-goat secondary antibody for 1 h at room temperature. Peroxidase activity was revealed by incubation with diaminobenzidine tetrahydrochloride (DAB) and H_2O_2 (Vector Lab. Inc., CA, USA). After washing, sections were weakly counterstained with hematoxylin. As negative control, primary antibody was replaced by donkey serum. Specificity of primary antibody had been previously assessed on human skin histological sections subjected to immunohistochemical reactions as above. Observations and photographic records were made using a Reichert-Jung Polyvar light microscope.

Immunogold labelling detection of IL-ra

Ultrathin sections from lot 2 samples were incubated for 10 min in 0.05M TRIS-HCl buffer, pH 7.6, added with 0.1% BSA. After incubation with 5% normal

donkey serum for 30 min, ultrathin sections were incubated with goat anti-human 1:25 diluted IL-1ra polyclonal antibody, overnight at 4°C. After washing with the same buffer, ultrathin sections were incubated with biotinylated donkey anti-goat secondary antibody (Amersham Ltd, Buckinghamshire, UK), for 1h. Subsequently, ultrathin sections were incubated with 0.02M TRIS-HCl buffer, pH 8.2, added with 0.1% BSA, for 10 min, and finally with 10nm gold-conjugated streptavidin (Amersham Ltd, Buckinghamshire, UK), for 1h. After washing, sections were weakly contrasted with uranyl acetate and lead citrate. As negative control, primary and/or biotinylated secondary antibodies were omitted during sample processing. Specificity of primary antibody had been previously assessed on human skin ultrathin sections subjected to immunogold labelling as above. Observations and micrographic records were made using a Philips CM12 STEM transmission electron microscope.

Results

Light microscopy

In normal heart samples, myocardium displayed weak IL-1ra immunoreactivity at level of cardiomyocytes, whereas the cytokine was not present in the interstitium (Fig. 1A).

Concerning infarcted heart samples, nonischemic myocardium areas exhibited positivity patterns comparable with those in normal myocardium, except for little more marked reactivity exhibited by myocytes (Figs. 1B,C, 2B).

In the border zone of the infarction, cardiomyocytes showing coagulation necrosis to early coagulative myocytolysis features were characterized by increased positivity for IL-1ra. The extracellular matrix was generally negative or weakly positive in places (Figs. 1D,E, 2B).

After picro-Sirius red staining, samples from ischemic zones showed areas occupied by ipervascularized dense connective tissue, as well as areas where pale, small cardiac muscle cells affected by prominent loss of cytoplasmatic substance and intercellular contacts were interspersed within abundant extracellular matrix (Fig. 2A). In addition to such damages correlating with advanced coagulative myocytolysis, these cardiomyocytes also disclosed strong reactivity for IL-1ra (Figs. 2B, 1F,G). Moreover, remarkable immunoreactivity was apparent for the extracellular matrix and few interstitial cells likely corresponding to neutrophils and macrophages (Figs. 1F-H, 2B). Interestingly, connective tissue immunoreactivity was more evident near blood vessels and cardiomyocytes. Conversely, no immunoreactivity was detected for vascular smooth muscle cells (Fig. 1F) and endothelial cells (Fig. 1D,F,H), even though IL-1ra antibody stained residual plasma in blood vessel lumens in the ischemic areas (Fig. 1F,H).

Electron microscopy

On ultrathin sections, immunogold labelling reactions for IL-1ra were consistent with the distribution patterns displayed by light microscopy.

In normal heart samples, weak reactivity for IL-1ra was restricted to cardiomyocytes, in which a moderate gold particle distribution was apparent with preferential localization at level of the intercalated discs (Fig. 3A), whereas no IL-1ra-immunopositivity was shown by noncardiac cells and extracellular matrix.

In infarcted heart samples excised from non-ischemic areas, similar labelling was observed as above (Fig. 3B), except for a slightly increased presence of metal particles on the muscular fibers and intercalated discs, with addition of Z-lines as preferential labelling sites, even if barely perceivable.

In samples excised from both border zones and ischemic areas of the infarction, remarkable intracellular immunoreactivity for IL-1ra was shown by injured cardiomyocytes.

In cardiomyocytes seemingly involved in initial coagulation necrosis, gold particles were particularly abundant at level of thickened Z-lines (Fig. 3C) and intercalated discs (Figs. 3C,D), which were often characterized by multiple, overgrown interdigitations (Fig. 3D).

Additionally, gold particles decorated plasmamembrane tracts as well as degenerating mitochondria and heterogeneous, rounded organule remnants resulting from coagulative myocytolysis progression in cardiomyocytes (Fig. 4A).

In ischemic areas, immunoreactivity for IL-1ra was still present in cardiomyocytes which resembled cell "ghosts" because of dramatic loss of the contractile component depending on protracted myocytolysis (Fig. 4B-D). Moreover, in the extracellular matrix, gold particles were mainly localized at correspondence with collagen fibrils, elastin fibres and fibrillin microfibrils (Fig. 4A-D). IL-1ra-immunoreactive extracellular matrix components were mainly found near cardiomyocytes and blood vessels.

Discussion

The involvement of IL-1ra in myocardial infarction and its beneficial role in limiting IL-1-mediated cardiac dysfunction have been shown to occur in several animal models of I/R injury (Suzuki et al., 2001; Chimenti et al., 2004). However, experimental conditions show some limitations. First, some unavoidable differences will exist between the effects of induced I/R injury in animal models and what actually happens in naturally occurred myocardial infarction in humans. Second, IL-1ra abundance depends on experimental administration in animal models, thus unabling to determine which is/are the effective source/s of IL-1ra in organs and tissues.

IL-1ra involvement in counteracting IL-1-mediated deleterious effects was also reported for human

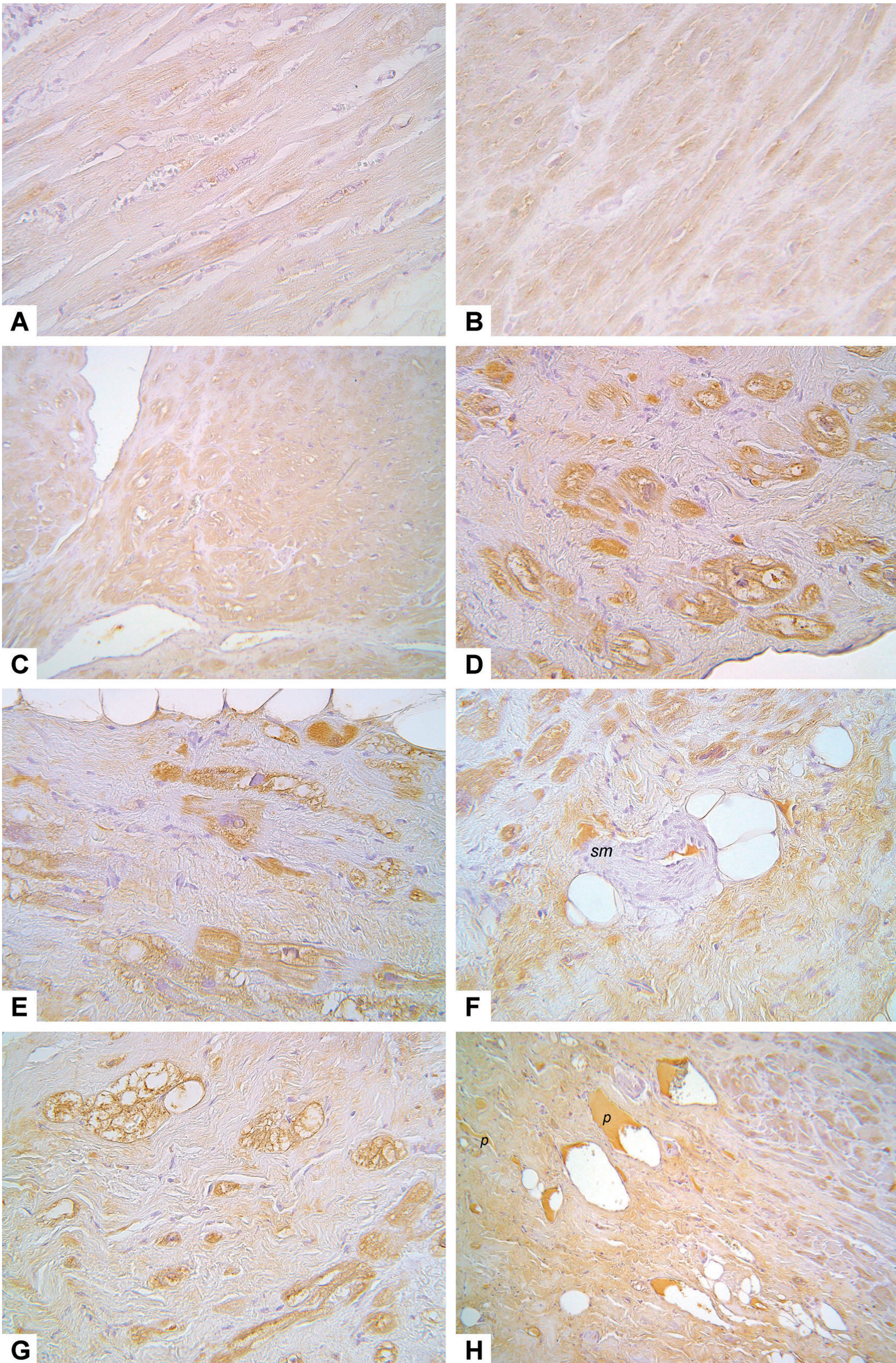

Fig. 1. Immunohistochemical detection of IL-1ra in normal and infarcted myocardium. **A.** Myocardium sample from normal heart. **B, C.** Nonischemic myocardium samples from infarcted heart. **D, E.** Infarcted heart samples: damaged cardiomyocytes from border zone of infarction (coagulation necrosis and/or early coagulative myocytolysis). **F.** Ischemic area sample: reactivity for damaged cardiomyocytes (advanced coagulative myocytolysis) and extracellular matrix and unreactivity for vascular smooth muscle cells (sm). **G.** Ischemic area sample: immunopositivity for injured cardiomyocytes (advanced coagulative myocytolysis). **H.** Ischemic area sample: immunoreactivity for intravascular plasma (p) and extracellular matrix. A, B, D-G, x 20; C, H, x 8

myocardial infarction because of the assessment of raised plasmatic IL-1ra levels in patients undergone ischemic episodes (Latini et al., 1994; Shibata et al., 1997; Patti et al., 2004). In a first attempt to explain this phenomenon, it has been suggested that circulating IL-1ra may act as an acute phase protein secreted by hepatocytes (Gabay et al., 1997). Despite IL-1ra was subsequently reported to be a natural anti-inflammatory protein produced by many other cells (Arend et al., 1998; Arend, 2002), such an involvement was still unexplored for cardiomyocytes. In this study, therefore, the possibility was investigated that myocardium could be a source of IL-1ra, and the first evidence that noninfarcted cardiomyocytes constitutively produce IL-1ra has been achieved. Since IL-1ra seems to balance the IL-1-mediated effects not only in pathological conditions but also in physiologic ones (Arend, 2002), it is likely that basal production of IL-1ra can normally occur in healthy cardiomyocytes. Moreover, IL-1ra production resulted to undergo a significant increase during myocardial infarction, being IL-1ra immunoreactivity progressively more intense moving from nonischemic toward ischemic areas of infarcted myocardium.

In particular, the main source of IL-1ra was identified in cardiomyocytes affected by either coagulation necrosis or coagulative myocytolysis in the ischemic and border areas of infarction, whereas only faint IL-1ra-positivity was detected for cardiomyocytes populating the nonischemic areas.

Taking in account that apoptotic cell death occurs in

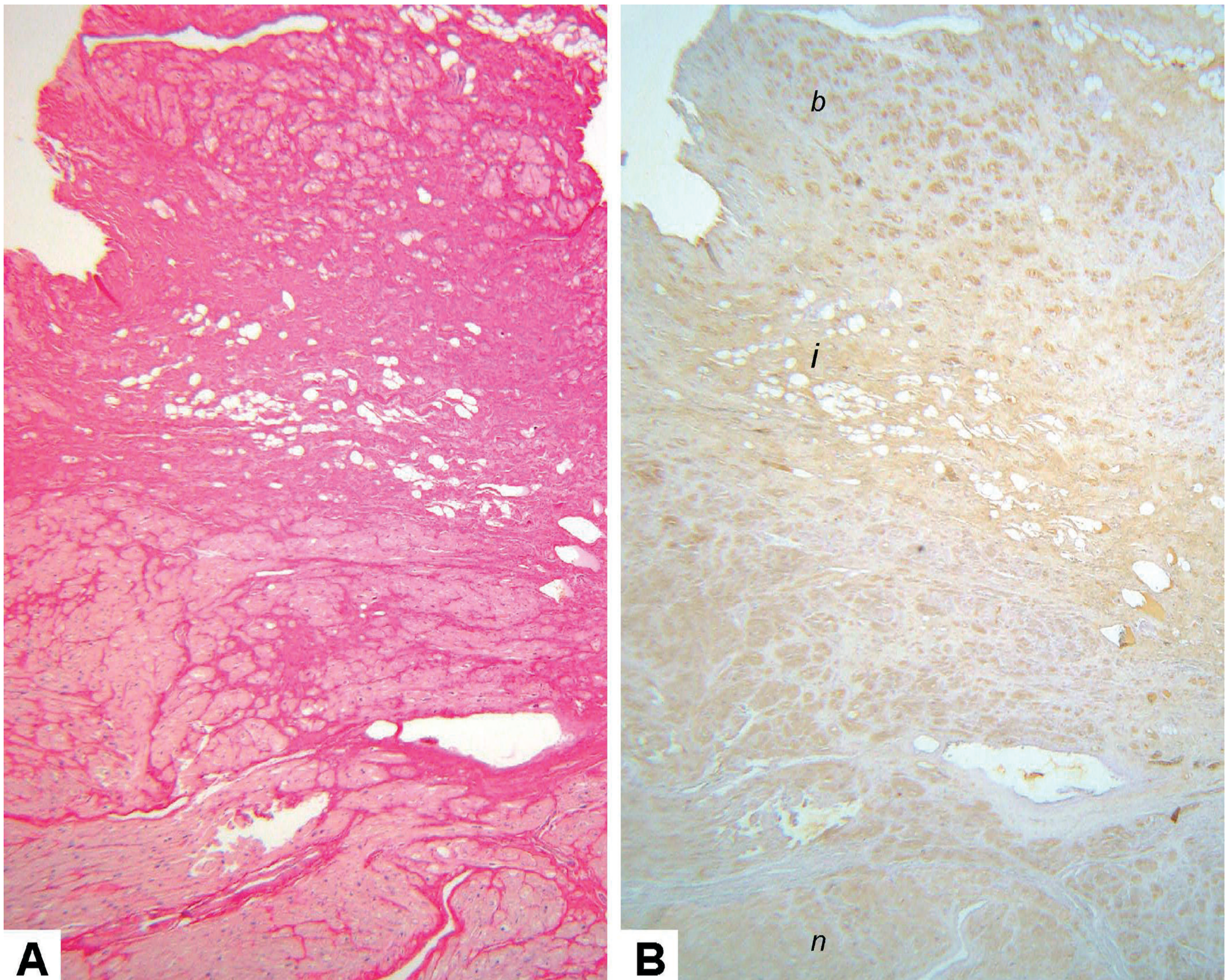

Fig. 2. Low magnification of a human infarcted heart sample showing myocardial nonischemic (n), ischemic (i), and border zones (b). **A.** Picro-Sirius red and hematoxylin staining. **B.** Immunohistochemical reaction with goat anti-human IL-1ra polyclonal antibody and counterstaining with hematoxylin. x 3

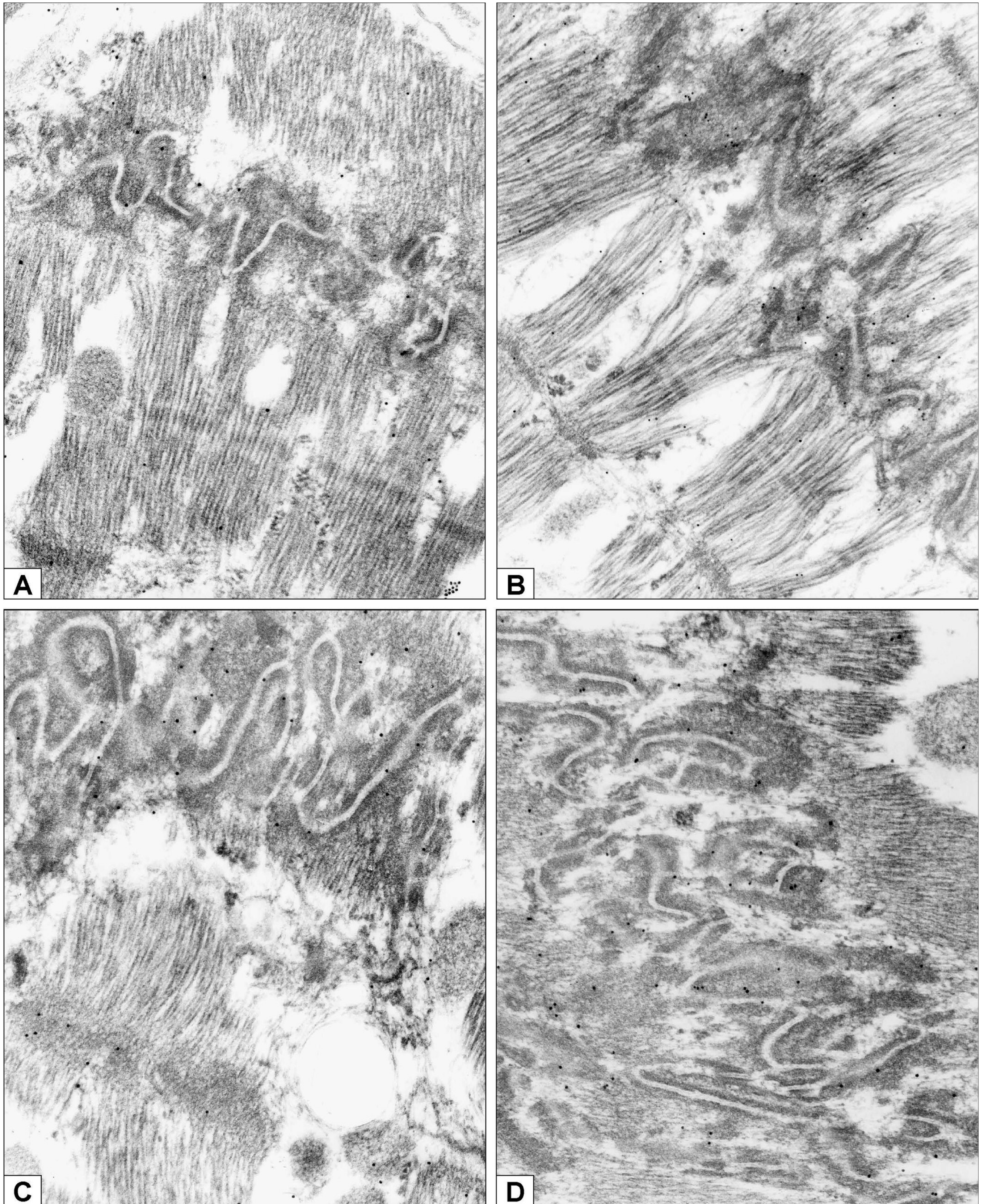

Fig. 3. Immunogold labelling with anti-IL-1ra Ab in normal and infarcted heart samples. **A.** Myocardium sample from normal heart: preferential distribution of gold particles on an intercalated disc. **B.** Myocardium sample from a non-ischemic zone of infarcted heart: as for (**A**), with slightly higher gold particle distribution. **C, D.** Myocardium samples from ischemic zones of infarcted heart: generalized increase in gold particle distribution with preferential propensivity exhibited by thickened Z-lines (**C**) and multiple intercalated discs (**C, D**). A, D, x 11,000; B, x 8,500; C, x 10,400.

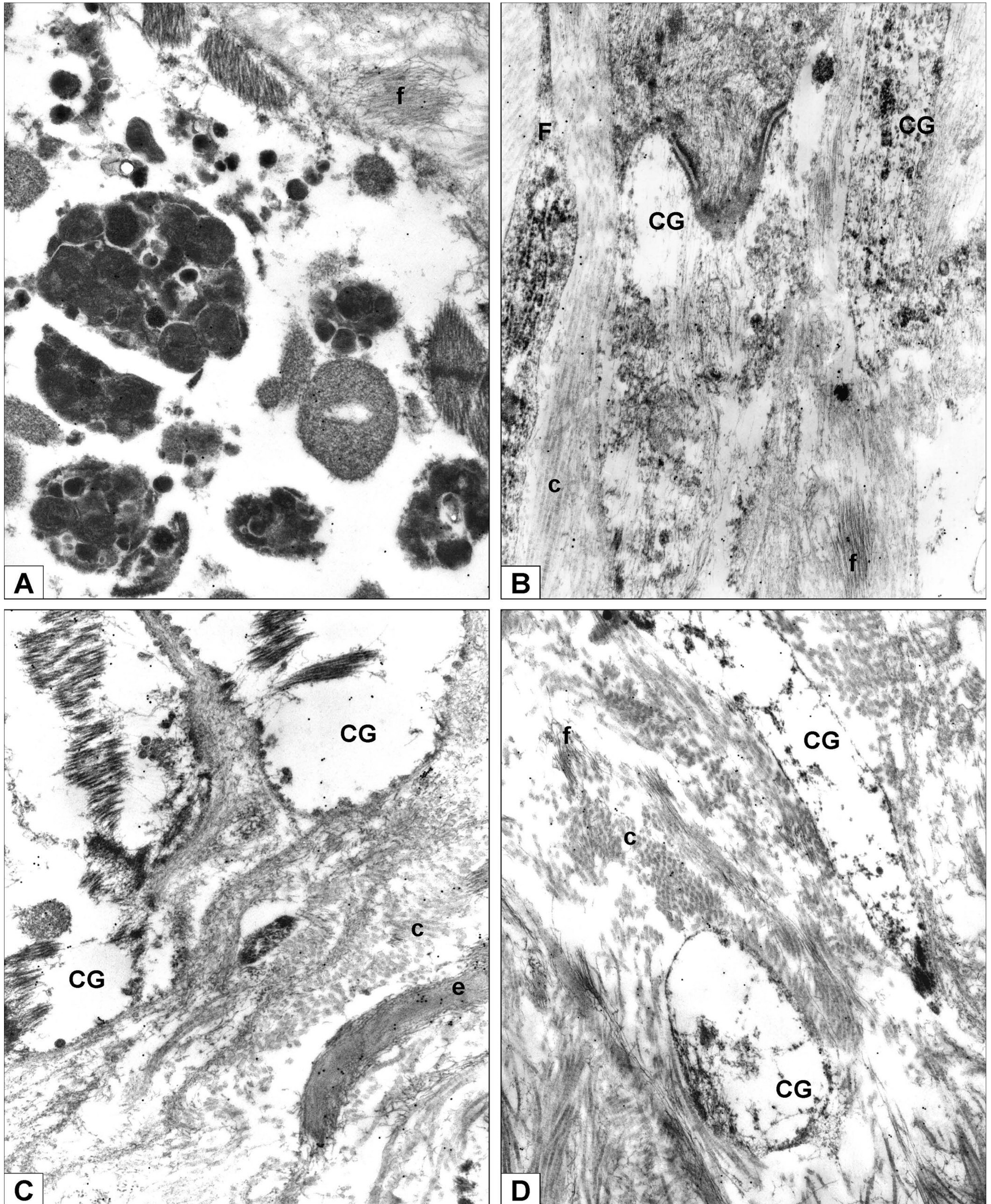

Fig. 4. Immunogold labelling with anti-IL-1ra Ab in infarcted heart samples. **A.** Gold particles on dense body-like structures and degenerating mitochondria in ischemic cardiomyocytes. Gold particles on extracellular matrix components (fibrillin microfibrils (f)). **B-D.** Gold particles on cardiomyocyte ghosts (CG). Gold particles on extracellular matrix components, collagen fibrils (c), elastic fibers (e) and fibrillin microfibrils (f). (F: fibroblast). A-C, x 18,500; D, x 14,000.

acute myocardial infarction (Saraste et al., 1997; Yaoita et al., 2000; Akasaka et al., 2006) and that IL-1 expression correlates with apoptosis (Yaoita et al., 1998), it is likely that the observed IL-1ra production increase can be ascribed to an attempt by cardiomyocytes to contrast IL-1-induced apoptosis.

In ischemic myocardium, cardiomyocytes are known to be characterized by features comparable with those in cardiac hypertrophy, such as formation of multiple intercalated discs, Z-line thickening, and degenerative changes including loss of contractile elements and intercellular connections (Maron and Ferrans, 1978).

In the present work, the observed ultrastructural damage features, such as thickened Z-lines and intercalated discs showing multiple, overgrown interdigitations, are consistent with those previously reported for ischemic myocardium. Additional information is represented by the relation found between the onset and progression of cardiomyocyte alterations and progressive increasing in immunogold labelling for IL-1ra. Putatively, the increased propensity to this immuno-labelling by multiple intercalated discs and thickened Z-lines might indicate that this protein plays some protective role for cardiomyocytes undergoing hypertrophic processes.

Since these two sites are coincevably subjected to the strongest mechanical forces, it could be speculated that one of the ways by which IL-1ra exerts its cardio-protective ability consists in a stabilizing role as regards the connection between contractile apparatus and intercalated discs and costameric machinery. Consistently, in cardiomyocytes the costameric protein complexes involved in mechanotransduction between cytoplasmic contractile filaments and extracellular matrix components closely interact with the outer Z-lines (Danowsky et al., 1992; Samarel, 2005).

Interestingly, some evidences suggest the existence of other relationship between IL-1ra and myofilament rearrangement in skeletal muscle. In particular, in animal models of sepsis it has been reported that infusion of IL-1ra can prevent infection-induced inhibition of myofilament protein synthesis (Zamir et al., 1994; Vary et al., 1996).

Despite the antibody used in this study could not discriminate between IL-1ra secreted isoform and intracellular ones, it is likely that cytoplasmic IL-1ra revealed in normal and nonischemic cardiomyocytes corresponds to an intracellular isoform since no immunoreactivity was detected at level of endoplasmic reticulum or secretory vesicles as well as in the extracellular matrix. As well, in border areas of infarcted myocardium, the more intense positivity for IL-1ra in cardiomyocyte cytoplasms might correlate with an increase in intracellular IL-1ra production. Actually, no or negligible IL-1ra-positivity was shown by the extracellular matrix in these areas, as for normal and nonischemic myocardium.

The stronger IL-1ra positivity found in ischemic areas could depend on increased intracellular IL-1ra production, whereas the presence of reactivity in the extracellular matrix could be ascribed to additional production and release of secreted IL-1ra or abnormal release of intracellular IL-1ra due to plasmamembrane alteration in cardiomyocytes undergone coagulative myocytolysis.

The preferential deposition of colloidal gold at level of collagen fibrils, elastin fibers and bundles of fibrillin microfibrils is hardly explainable but represents an intriguing issue. Direct interaction has been reported between different cytokines and glycosaminoglycans. In particular, concerning interleukin family members, the finding of possible interaction of interleukin-2 with heparan sulphate glycosaminoglycans was interpreted as a mechanism by which this cytokine is retained near its secretion sites (Najjam et al., 1997). Temptatively, it could be speculated that IL-1ra might bind indirectly the matrix fibrous components as above via glycosaminoglycans. Since IL-1ra involvement in myocardium is still far to be elucidated, such an assumption could serve just as a starting point for future investigation.

Apart from possible involvement of the extracellular matrix in ischemic areas, its reactivity with increasing around altered cardiac cells can be correlated to IL-1ra release by these cells, thereby seeming major cardiac source of this cytokine. Such conclusion is corroborated by the absence of significant IL-1ra immunostaining observed for interstitial cells.

Further, IL-1ra-immunopositive endothelial cells were not detected in both normal and infarcted myocardium. However, it cannot be excluded that blood vessels may represent an additional source for IL-1ra consistently with the finding that both intracellular IL-1ra and secreted IL-1ra are naturally expressed *in vivo* by ischemic coronary endothelial cells (Dewberry et al., 2000).

Thus, it might be assumed that in infarcted hearts endothelial cells produce IL-1ra secondary to atherosclerotic degeneration of ischemic blood vessels in spite of the fact that endothelial cells did not show IL-1ra positivity, because of its release as secreted isoform. Moreover, it is possible that during infarction circulating IL-1ra could diffuse into neighbouring myocardial tissue *via* vessel wall endothelia. Such hypothesis is consistent with the IL-1ra positivity exhibited by perivascular extracellular matrix in ischemic areas of infarcted myocardium. In the same areas, the detected plasma reactivity could be ascribed to aspecific positivity; however, in normal myocardium no reactivity was detected for plasma and actual IL-1ra presence in plasma and leucocytes was shown (Latini et al., 1994; Shibata et al., 1997; Malyak et al., 1998; Arend and Guthridge, 2000; Patti et al., 2004). Likewise, vascular smooth muscle cells did not show IL-1ra-positive staining in both noninfarcted and infarcted myocardium. As was hypothesized for endothelial cells, it is conceivable that in ischemic areas of infarction vascular smooth muscle cells can secrete IL-1ra not intracellularly detectable

because of its possible release into the neighbouring extracellular matrix, as suggested by the finding that IL-1ra is present in supernatants of opportunely stimulated vascular smooth muscle cell cultures (Di Febbo et al., 1998).

Despite of the multiple putative IL-1ra sources immunohistochemically revealed in infarcted myocardium, it is obvious that critical conditions can take place in which increased IL-1ra levels are not sufficient to effectively block the biological effects of the locally produced IL-1, as was observed in other inflammatory diseases such as arthritis (Arend, 2002) or atherosclerosis (Arend and Gabay, 2000).

In conclusion, the present results strongly suggest that a distinct IL-1ra distribution pattern exists in myocardium advising the existence of intrinsic production sites, and represent a starting point for further investigation. Additional work has been undertaken to confirm the identity of this protein and to gain more detailed information on its involvement in normal and infarcted myocardium.

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