Localization of the apoptosis repressor ARC and anti-apoptotic factor FLIP in endomyocardial biopsies of cardiomyopathy patients

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SUMMARY

Endomyocardial biopsies from patients with dilated cardiomyopathy were obtained by cardiac catheterization. Tissue samples were embedded in epoxy resin and sectioned into serial semithin sections for immunhistochemical visualization of either the apoptosis repressor protein or the FLICE inhibitory protein. Colocalization of apoptosis repressor protein and FLICE inhibitory portein was observed in cardiac myocytes, with nuclear DNA fragmentation and myofibrillary degradation. Intact myocytes were devoid of FLICE inhibitory protein and apoptosis repressor protein staining. FLICE (Fas-associated death-domain-like IL-1 -converting enzyme) inhibitory protein and apoptosis repressor protein were localized in the perinuclear cytoplasm, mainly in areas that showed myofibrillary degradation as visualized by fluorescence microscopy. FLICE inhibitory and apoptosis repressor proteins may be intracellular markers for monitoring myocardial damage in several cardiac diseases in humans.

Key words: Heart – Apoptosis – Cardiomyopathy – Contractile apparatus – Myocytes

INTRODUCTION

ARC, Apoptosis Repressor with a Caspases recruitment domain (CARD) is a protein known to be expressed in human skeletal muscle and myocardium in the course of programmed cell death (Koseki et al., 1998). ARC has been shown to inhibit the enzymatic activity of caspases 2 and 8 whilst not interacting with the function of caspases 1, 3 and 9 (Thornberry et al., 1992; Yuan et al., 1993; Salvesen and Dixit, 1997; Koseki et al., 1998). ARC has been reported to attenuate apoptosis induced by FADD, TRADD and stimulation of death receptors coupled to caspase 8, such as CD95/Fas, TNF-R1 and TRAMP/DR3 (Boldin et al., 1996; Muzio et al., 1996; Chinnaiyan et al., 1996).

Two mechanisms by which ARC inhibits apoptosis have been described so far: (1) Its CARD binds to the death effector domains (DED) of FADD and caspase 8, thus inhibiting the interaction of these two proteins . (2) ARC prevents the release of mitochondrial cytochrome C into the cytoplasm, which would usually happen during some forms of cytotoxic stress, and would prevent the consecutive engagement of the apoptotic cascade through caspase 9 (Koseki et al., 1998; Li et al., 1995). Bcl-2 and Bcl-XL, inhibitors of apoptosis found in most types of tissue, are not expressed in large amounts in myocytes. It is therefore possible that ARC might be their functional equivalent in these tissues (Rudel, 1999; Matsuda et al., 1995).

FLIPs, the FLICE- (Fas-associated death-domain-like IL-1 -converting enzyme) inhibitory proteins, selectively inhibit the function of cas-

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pase 8 (Irmler et al., 1997). The mRNA of FLIP is highly expressed in skeletal muscle and myocardium, most likely protecting these tissues from Fas-induced apoptosis (Irmler et al., 1997). However, despite the presence of high FLIP mRNA levels, in many cases anti FLIP reactive protein cannot be detected in many cases. This indicates that FLIP expression may be controlled at post-transcriptional level. Low protein expression rates of FLIP (also called Usurpin by Rasper et al., 1998) have been observed in myocytes undergoing apoptosis upon myocardial infarction after ischemia or reperfusion injury (Rudel, 1999,), whereas in "healthy" human and rat cardiomyocytes strong immunostaining for FLIP (Usurpin) has been oberserved (Rasper et al., 1998). FLIPs are highly homologous to caspase-8 but lack its catalytic activity, thus inhibiting it competitively and hence disrupting the signal transfer from the Fas/CD95-receptor (Salvesen and Dixit., 1997; Thome et al., 1997).

Endomyocardial biopsies have gained increasing importance for the assessment of myocardial ailments, including idiopathic dilated cardiomyopathies (DCM) (Messam and Pittman, 1998). Several morphological and histochemical methods have been employed to differentiate the types and status of myocardial diseases (Kandolf, 1998; Richardson et al., 1998; Sigusch et al., 1998). In this sense it became evident that apoptosis may play a significant role in chronic heart failure due to ischemia or chronic viral infections. However, the actual time-course of apoptosis in different tissues is still a matter of controversy, and in DCM it is not known (Aschoff and Jirikowski 1997; Li et al., 1996; Messam and Pittman, 1998). The evaluation of myocardial damage prior to actual apoptosis might be a useful tool for monitoring different treatments of DCM.

In a recent study we used semithin sections of resin-embedded biopsies to determine both nuclear DNA fragmentation and immunoreactivity for tissue transglutaminase (Aschoff et al., 2000). With this technique it is possible to stain consecutive 0.5 µm thick sections with different antibodies, allowing colocalization studies to be carried out at single cell level (Aschoff & Jirikowski, 1997; Aschoff et al., 1999). In the present paper we used this technique for the visualization of $FLIP_S$ and ARC in human cardiomyocytes, combined with staining for nuclear DNA-fragmentation and myofibrillary autofluorescence.

Materials and Methods

Samples were obtained from patients with idiopathic dilated cardiomyopathy who had undergone cardiac catheterisation to collect a right or left ventricular endomyocardial biopsy. After removal, the specimen were immediately fixed in 5% paraformaldehyde in 0.1 M phosphate buffered saline (PBS). After dehydration, samples were embedded in EPON resin and sliced into 1µm serial semi-thin sections on a Reichert Ultracut microtome. Prior to immunostaining, the resin was removed with sodium methoxide and sections were rehydrated as previously described (Aschoff et al., 1996). All Techniques used in the investigation complied with the principles outlined in the Declaration of Helsinki.

For the visualization of ARC and $FLIP_S$, subsequent semithin sections were incubated with either polyclonal goat anti-ARC or goat anti-$FLIP_S$, (both obtained from Santa Cruz, USA) at a dilution of 1:200 in PBS, containing 3% rabbit serum. After 12 h incubation at 4°C, the slices were rinsed in PBS and then treated with a 1:200 solution of biotinylated rabbit anti-goat IgG for 1 h at 20°C. Then, Streptavidin-HRP (1:200) was applied for 1 h at 20°C. Sections were washed twice in PBS at RT for 10 min. after each incubation step. For visualization of immunoprecipitates, diamidinobenzidine (DAB) and H_2O_2 were used.

For in-situ end-labeling with bromodeoxyuridine (BrdU), subsequent sections of the ARC- and $FLIP_S$- stained sections were rehydrated as described above. Sections were incubated with terminal transferase, diluted in tailing buffer (Boehringer, Mannheim) and 5'bromo-2'desoxyuridine (BrdU, Sigma, München). After washing, mouse anti-BrdU (Progen, Heidelberg) was used followed by goat anti-mouse IgG (1:100) at 20°C for 1 h, after which mouse monoclonal PAP (1:100) was applied for 1 h at 20°C. Sections were washed twice in PBS at RT for 10 min. after each incubation step. Following this, DAB was used to visualize DNA strand breaks. For details on this method, see (Aschoff et al., 1996). For immunocytochemical controls, sections were incubated with a rabbit normal serum, at the same dilution as the specific primary antibody. Such sections were processed along with the other staining reactions. Stained series of sections were studied in an Olympus BX 50 photomicroscope under interference contrast illumination. For observations of overall morphology, unstained sections were subjected to epifluorescence with the WU filter combination, in order to observe myofibrillary autofluorescence.

Results

Due to the technique of staining consecutive semi-thin serial sections, we were able to co-localize different proteins at single cell level. Healthy tissue showed strong auto-fluorescence, implying an intact myofibrilliary structure (Fig 1A, bright blue, and fig. 2A, greenish color), although in some cases nuclei with fragmented DNA could be detected in these cells. In all pic-

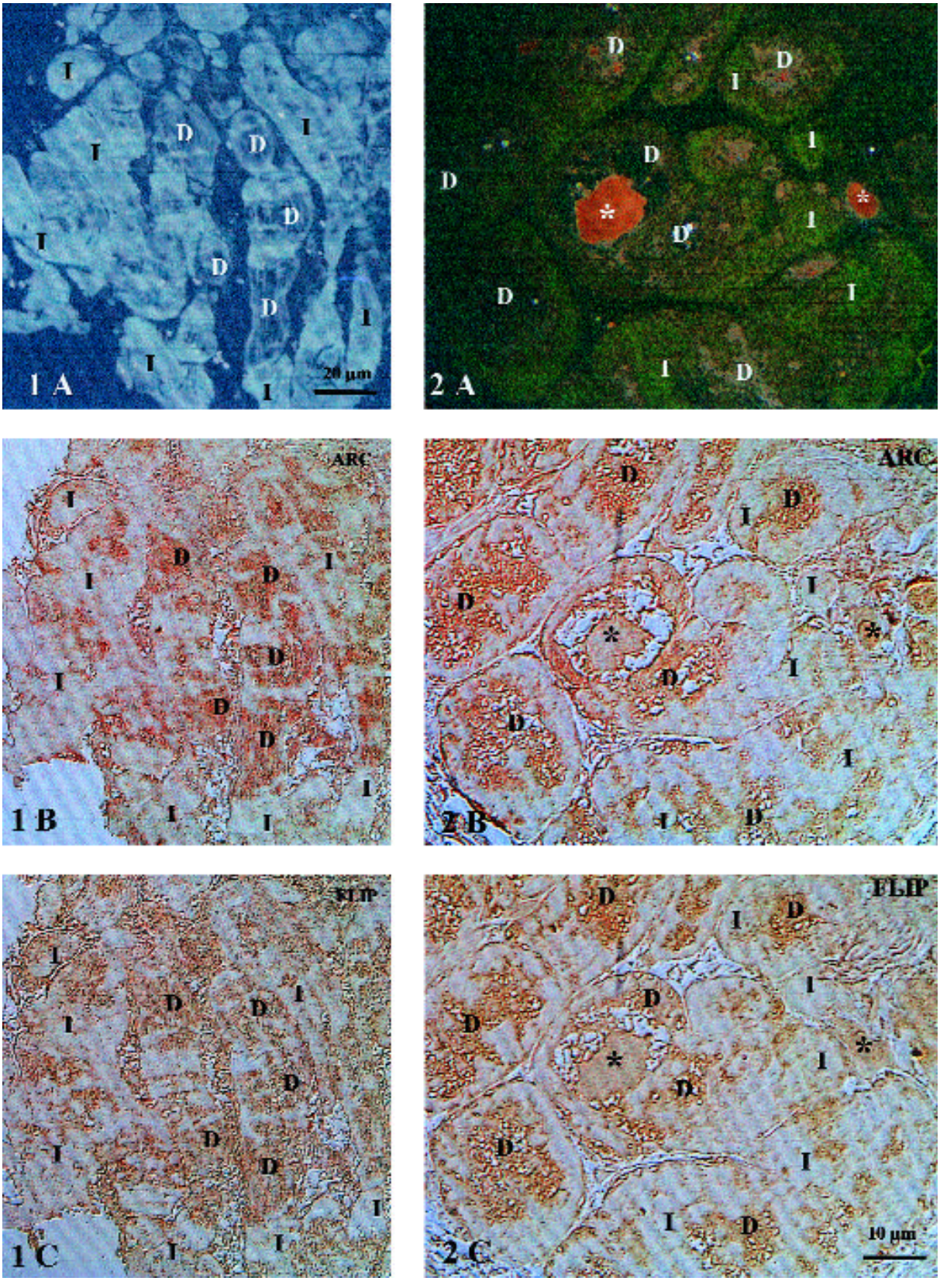

Figure 1.- Consecutive semi-thin sections of myocardial biopsy from a DCM patient (400x). 1A: Epifluorescence image demonstrates the loss of auto-fluorescence in some of the cells, an indicator of cell damage (marked with D). Other cells show strong bright blue autofluorescence, and indicator of intact cells (marked with I). Comparison with the consecutive sections 1B and 1C (bright field) reveals an accumulation of ARC and of FLIPs in the damaged areas, and the absence of these proteins in the cells or areas marked with I.

Figure 2.- Consecutive semi-thin sections of myocardial biopsy from a DCM patient (800x). 2A: Epifluorescence image shows a cell with severe damage to its myofibrillar structure and a nucleus showing heavy DNA-fragmentation (brown, marked with an asterisk). Loss of auto-fluorescence appears brown or grey in contrast to intact cytoplasm, which appears as green. Damaged areas are marked with D; intact cells or areas with I: Lysosomes appear as small white spots. Cytoplasmic immunoreactivities for ARC (Fig. 2B) and for FLIPs (Fig. 2C) occur in identical locations; the intracellular distribution of both proteins however, is different. The loss of auto-fluorescence and the high levels of ARC and FLIPs can be seen particularly in the center of the myocytes, while many cells show an intact periphery.

tures, intact cells or intact areas are marked with an **I**, and damaged cells with **D**. By comparison with ensuing sections stained either with ARC or FLIP, it was evident that in intact cardiomyocytes immunostaining for these proteins was very low (Figs 1B, 1C and 2B, 2C).

Fig. 1 shows sections from a biopsy at low magnification (400x), taken from a DCM patient. They reveal the typical histological phenotype of a heart suffering from dilated cardiomyopathy, some cells appearing stretched and having bizarre shapes. In damaged cells, a significant loss of auto-fluorescence is evident (Fig. 1A). At higher magnifications damaged cells (Fig. 2A) are characterized by a greyish cytoplasm with small bright dots, and highly disintegrated structures in the perinuclear cytoplasm. In these cells, nuclei showing heavy DNA-fragmentation were always found (Fig, 2A, asterisks). In other cardiomyocytes with an intact myofibrillar structure, nuclei with fragmented DNA were also occasionally seen. A comparison of ARC (1B, 2B) and FLIPs (1C, 2C) staining shows that the accumulation of these proteins is abundant in damaged areas, where autofluorescence is attenuated. Although immunohistology by anti-ARC antibodies proved to be more intense and appeared more widespread than staining with FLIPs, it is evident that both proteins are localized in the same areas of the cell.

In our biopsies, about 20% of cells were stained with BrdU as an indication of DNA-fragmentation. Many of the cardiomyocytes with fragmented DNA showed significant accumulations of ARC and FLIPs (Fig. 2), although only in those cells with reduced auto-fluorescence, an indicator of myofibrillar disintegration. Both proteins were prefentially localized in damaged areas of cardiomyocytes, whereas the peripheral parts, often with intact auto-fluorescence, showed low reactivity to ARC or FLIPs. In the severely damaged region around the nucleus the intensity of immunoreactivity for ARC and FLIPs was attenuated. Our findings demonstrate that ARC and FLIPs are predominantly found in those parts of the myocardial cytoplasm, where auto-fluorescence has been significantly reduced due to myofibrillar degradation, and not in intact cells.

DISCUSSION

The correlation of myofibrillar loss and DNA-fragmentation argues for significant cellular damage and probably the beginning of apoptosis. The correlation of reduced autofluoescence with myofibrillar desintegration and cellular damage has been reported previously (Busch et al., 2001). The fact however, that in cardiac tissue, so many cells can be found showing DNA-fragmentation and no myofibrillar degradation indicates that actual cell death has not yet taken place in these cases. We were able to detect ARC and FLIPs only in damaged cardiomyocytes, which is at first sight contradictory to the proposed protective functions of ARC and FLIPs. It is also in clear contradiction with the findings of Rasper et al. (1998) who found strong FLIPs immunoreactivcity in “healthy” rat cardiomyocytes and reduced immunoreactivity in ischemia-damaged cells.

One possible explanation is that the expression of these proteins would be triggered by noxious stimuli before actual apoptosis takes place, similar to the up-regulation of heat-shock proteins. This would explain why we detected ARC and FLIPs in damaged cardiomyocytes but not in undamaged cells. The difference with the results of Rasper et al. (1998) might be explained by the method of tissue preparation, the interpretation of the immunostaining results and by the kind of tissue used. Rasper et al. (1998) used paraffin sections (probably 10-µm thick sections) of rat heart and localized immunoreactivity of FLIPs after experimental ischemia mainly in experimentally undamaged regions of the heart. The conclusion that cardiomyocytes in these sections were really undamaged was based the fact that no DNA-fragmentation was detected, a conclusion that due to our findings seems not to be justified. Rasper et al. (1998) found that in regions of “healthy” cardiac tissue, no DNA-fragmentation could be detected with the TUNEL method. This controversy can easily be explained by the sensitivity of the different methods used. Our ISEL technique on semi-thin sections is probably 100 times more sensitive than the TUNEL technique on paraffin sections (Aschoff and Jirikowski, 1997). Thus we were able to detect “natural” or non-enzymatic DNA-fragmentation, probably caused by oxidative stress, whereas the TUNEL technique detects only or mostly massive DNA fragmentation, caused by enzymatic digestion of the genome (Aschoff et al., 2000). Moreover, due to the low resolution of the method used, Rasper et al. (1998) failed to detect the distribution of FLIPs within single cardiomocytes. Our tissue was human tissue and we used semi-thin sections (0.5 µm thick), identifying individual cells. Thus, we were able to localize FLIPs and ARC in damaged regions within a single cell. We observed that ARC and FLIPs are only expressed in cardiomyocytes that already show some kind of cellular damage: i.e. DNA-fragmentation and reduced auto-fluorescence. It is most likely that this “cellular damage” is not yet apoptosis and even more likely that these damaged cells reside in the tissue for a long time. This may be because damaged cardiomyocytes are able to express protective proteins, as is the case with Bl-2 and Bax in enterocytes of the small intestine

(Aschoff et al., 1999). Rasper et al. (1998) found an attenuation of immunoreactivity for FLIPs in experimentally damaged regions of the rat heart. In these regions, they found high DNA-fragmentation and strong immunoreactivity for Caspase-3, clearly an indicator of apoptosis. This is in agreement with our findings that in heavily damaged cells, with a disintegrated cytoplasm and strong DNA-fragmentation, the expression of FLIPs and ARC is attenuated.

Our results show that FLIPs and ARC are expressed only in cardiomyocytes that show signs of cellular damage, but there is no indication that these cells are prone to undergoing apoptosis. It is quite conceivable that in cases of severe acute damage these proteins are not expressed, and digestion by caspases and DNases predominates, leading to apoptosis or necrosis. Thus, the immunocytochemical detection of "protective" proteins such as ARC and FLIPs may become a valuable tool for the diagnosis of several heart diseases and for the estimation of whether repair and/or recovery may still be possible.

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