Induction of early immediate genes and programmed cell death following cardioplegic arrest in human hearts

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Abstract

Objective: Under experimental conditions cardiac stress may induce early immediate genes. Of these, heat shock proteins like hsp 70 have been linked to preconditioning and cellular salvage. Protooncogenes like c-fos and c-jun act as transcription factors for other genes and may be involved in the regulation of programmed cell death. Methods: Patients, 30, undergoing elective coronary artery bypass grafting, received either cold antegrade St. Thomas II or Bretschneider or Hamburg cardioplegic solutions with ten patients in each group. Tissue from right atria was removed before cardiopulmonary bypass and following cardioplegic arrest and reperfusion. Tissues were examined by Northern blots, immunohistochemistry, and in situ nick-end labeling of fragmented DNA as evidence for programmed cell death. Results: There were no significant preoperative or operative differences between groups. Following cardioplegia and reperfusion, a significant induction of both protooncogene and heat shock protein 70 mRNA was observed. Whereas levels of hsp 70 were increased about two-fold in all groups (P < 0.05), induction of c-fos and c-jun was most pronounced following the Hamburg cardioplegic solution (P < 0.05 versus baseline and for differences to other groups). Induction on the protein level was confirmed using immunohistochemistry that furthermore, identified cardiac myocytes and endothelial cells being the cell types that expressed these genes. In contrast to prebypass samples, in situ nick-end labeling of fragmented DNA following cardioplegic arrest and reperfusion was positive, preponderately in subendocardial myocytes and endothelial cells. Conclusions: Cold cardioplegia is a potent stimulus for induction of the early immediate genes examined in human hearts. Increased expression of protooncogenes may be deleterious to cardiac myocytes as indicated by in situ nick-end labeling of DNA fragments. Differences in gene induction may add additional information for the evaluation of different cardioplegic strategies. © 1997 Elsevier Science B.V.

Keywords: Cardiac surgery; Apoptosis; Heat shock protein; Protooncogenes

1. Introduction

Under experimental conditions cardiac stress like hyperthermia, ischemia, hypoxia, metabolic changes or increased wall tension has been shown to induce early immediate genes [7,9,13,16,21,25]. Increased levels of their corresponding messenger-RNAs may be observed minutes to hours after initiation of stress. Of these early immediate genes, heat shock proteins like hsp 70 have been linked to preconditioning and cellular protection against external noxae [8,10,15,18], whereas the protooncogenes c-fos and c-jun are involved in cellular differentiation and programmed cell death or apoptosis [14,21,23,24]. In contrast to necrosis, which develops in an unregulated fashion involving inflammation and dis-
turbed homeostasis, apoptosis is a controlled physiological process without inflammation for clearance of redundant or damaged cells [6,24].

The regulation of these early immediate genes in the human heart is unknown. In particular, data on their expression following cardioplegic arrest and reperfusion in a clinical setting are lacking. The objective of this study was to investigate the expression of these genes in the hearts of patients undergoing elective CABG using different cardioplegic solutions. Additionally, cardiac tissues were examined for evidence of programmed cell death.

2. Patients and methods

The study protocol was approved by the institutional ethics committee on human research. Following informed consent, 30 patients scheduled for elective CABG with multiple grafts were randomly allocated to three groups for operation with different cardioplegic solutions. Anaesthesia was performed with sufentanil, etomidate, pancuronium and isoflurane. For cardiopulmonary bypass a membrane oxygenator was used with a priming of 1400 ml Ringer solution with the addition of 250 ml 20% mannitol. Moderate systemic hypothermia (30–32°C) was applied in all patients. Following aortic cross clamping, 100 ml of Kirsch solution (Cardioplegin, Kühler Chemie, Alsbach, Germany: 80 mmol/l Mg-aspartate, 11 mmol/l procaineHCl, 247 mmol/l xylitol) were injected into the aortic root immediately followed by antegrade infusion of Fresenius cardioplegic solution (Hamburg, Kühler Chemie, Alsbach, Germany) according to the Hamburg cardioplegia regimen [5]. Alternatively, two other groups received either St. Thomas II (ST) or Bretschneider solution (HTK; both obtained from Kühler Chemie, Alsbach, Germany). Composition of cardioplegic solutions as indicated by the manufacturers is shown in Table 1. Before infusion, all cardioplegic solutions were kept refrigerated at 4°C. Bretschneider solution was given in a single dose (30 ml/kg bodyweight), the other solutions repeatedly in 30–40 min intervals after the initial infusion of approximately 1 l. External cooling was performed by intrapericardial application of iced Ringer solution.

Small biopsies were taken from the right atrial appendage before cardiopulmonary bypass and after weaning from extracorporeal circulation. Immediately after removal tissues were processed for the following analyses.

2.1. Analysis of messenger RNA

Tissue samples were snap-frozen in liquid nitrogen and processed as previously described in detail [20,21]. Briefly, RNA was extracted with the cesium chloride method. Northern blot analysis, using the formaldehyde-agarose method, was carried out to separate mRNAs. Samples were blotted onto nylon filters and after a prehybridization period of 4 h hybridized overnight in a buffer with 32P cDNA probes [21]. Following multiple washings blots were exposed to X-ray films (Kodak XAR, Eastman Kodak, Rochester, NY, USA) for 24 h. Possible sample variability was checked for with a control cDNA probe of the constitutively expressed enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a recovery marker. Autoradiograms were scanned with a microdensiometer (LKB Instruments, Paramus, NJ, USA) for quantification of specific mRNA signals in relation to GAPDH separately in each sample.

2.2. Histological methods

Tissue samples were processed for immunohistochemistry to localize protooncogene and heat shock proteins. Following immersion in OCT 4583 (Miles, Elkhart, IN, USA) small samples were frozen in liquid nitrogen and processed for immunohistochemistry to localize protooncogene and heat shock proteins.
nitrogen and fixed after cryostat sectioning in cold (−20°C) acetone. Alternatively, tissue was prefixed in Bouins solution, rinsed in a 30% glucose solution and also frozen after immersion in OCT 4583. Cryostat sections were stained immunohistochemically with mouse monoclonal antibodies (anti-c-FOS, Medac, Hamburg, Germany; anti-HSP 72, Amersham Buchler, Braunschweig, Germany) according to the peroxidase-antiperoxidase-method using appropriate second and third antibodies (anti-mouse-IgG and monoclonal mouse-PAP-Komplex, both from Boehringer, Mannheim, Germany). Negative controls were performed by replacement of specific antibodies with non specific immunoglobulins.

In situ nick-end labeling of DNA fragments [11] (all reagents supplied by Oncor, Gaithersburg, M D, USA) was performed on tissue sections fixed in 10% buffered formalin. In brief, endogenous peroxidase was blocked with 2% hydrogen peroxide for 5 min. DNA fragment nick-end labeling with digoxigenin-dUTP was performed using terminal deoxynucleotidyl transferase by incubation for 60 min. Negative controls were incubated without this enzyme. Digoxigenin-dUTP was visualized after incubation with an anti-digoxigenin-peroxidase conjugate by 3,3-diaminobenzidine. Tissue from rat mammary glands and inflamed tonsils served as positive controls. Nuclear counterstaining was performed with methyl green. For better identification of cell types, adjacent sections were stained with hematoxylin and eosin.

2.3. Analysis of data

Results of mRNA analyses are expressed as ratio of early immediate genes to GAPDH. Data are presented as mean ± standard error. Non parametrical variables were compared using the Kruskal-Wallis test, parametrical variables by Student’s t-tests using Bonferroni corrections for multiple comparisons between groups. A probability value of less than 0.05 was considered indicative of a significant difference.

3. Results

No patient died perioperatively. There were no significant preoperative or operative differences between patient groups (Table 2). Cardioplegic arrest and reperfusion resulted in an induction of all examined early immediate gene mRNAs (Fig. 1). Heat shock protein 70 mRNA levels were increased about two-fold with all cardioplegic solutions compared with prebypass controls (P < 0.05). Cardioplegic arrest with the Hamburg cardioplegic solution and reperfusion resulted in approximately five-fold elevated levels of c-fos and c-jun. The increase of these protooncogenes was significantly higher in comparison to controls and the other cardioplegic solutions used. Following St. Thomas II and Bretschneider solution, the increase of c-fos was also significant as compared with controls, yet smaller than following HCS. For c-jun, there was a clear trend for increased post-arrest levels with these two solutions. However, it failed to reach the level of significance taking into account the multiple comparisons.

On the protein level immunohistochemistry with c-FOS antibodies displayed intense nuclear staining of endothelial cells, nuclei of cardiac myocytes were stained less homogeneously (Fig. 2). Similarly the inducible heat shock protein 72 was detected mainly in the nuclei and the cytoplasm of cardiac myocytes and endothelial cells (Fig. 3a). When specific antibodies...
were replaced by unspecific immunoglobulins, no staining was observed (Fig. 3b).

In situ nick-end labeling of fragmented DNA in tissue samples demonstrated positive staining mainly in the subendocardial layers (Fig. 4b). Additionally, the endocardium itself and other endothelial cells were marked. Inhomogeneous distributions were also observed between different patients receiving the same cardioplegic solution. Transmural serial countings of marked cells in high power visual fields and comparisons with adjacent sections stained with hematoxylin-eosin revealed a maximum of 2% of cardiac myocytes stained by nick-end labeling. In other patients, the average of stained cells was below 1%. In contrast, virtually no positive cells were found in control samples removed before cardioplegic arrest and reperfusion (Fig. 4a).

### 4. Discussion

Heat shock proteins have been linked experimentally to preconditioning and improved myocardial recovery following ischemia [10,15,18]. In a rat model of prolonged cardiac arrest after heat shock, induction of hsp 70 protein has been shown to parallel ventricular recovery [3]. Heat shock proteins assist in the folding and preservation of the tertiary structure of polypeptides in addition to their function as transcription factors [8]. All cold crystalloid solutions in our study with patients undergoing CABG resulted in a significant induction of hsp 70 mRNA. Previous data from our laboratory in isolated rat hearts indicated an important role of the type of cardioplegic solution [2]. Following cardioplegic arrest with ST and reperfusion, there was no change of hsp 70 mRNA levels, whereas HTK resulted in a five-fold increase. Aside from species differences, this may be explained by differences between the settings of isolated rat heart perfusion and the clinical situation, mainly collateral flow and blood reperfusion. The importance of blood in this respect is emphasized by findings that blood cardioplegia did not result in increased heat shock protein levels [19].

Relating to protooncogenes, however, we found significant differences with different cardioplegic solutions. Levels of c-fos and c-jun following the Hamburg cardioplegia doubled those after St. Thomas or Bretschneider solution. Since the latter two solutions are different in many respects, i.e. extracellular versus intracellular composition, electrolytes seem improbable as explanation for this phenomenon. However, the Hamburg cardioplegic solution is unique in its high content of hydroxyethyl starch. This results in a colloidosmotic pressure of 33 mmHg which at least matches serum values (normal > 23 mmHg) and highly exceeds the values of the other two solutions. Increased expression of c-fos has been found in isolated rat cardiomyocytes following hyperosmotic stress [26]. Therefore, the high osmolality and colloidosmotic pressure of the Hamburg solution may be responsible for the more pronounced induction of c-fos and c-jun we observed with this cardioplegia regimen.

We cannot exclude a possible influence of mechanical stress, ischemia or other factors on induction of the
examined early immediate genes in this clinical study. However, the different induction patterns observed with different cardioplegic solutions in patients and our findings in isolated rat hearts, where these mechanisms seem unlikely [2], stress the major impact of different cardioplegic strategies on expression of these genes. By immunohistochemistry we were able to localize the related proteins to nuclei and perinuclear area of cardiac myocytes and the vasculature and to confirm our data on mRNA expression on the protein level.

The protooncogenes c-fos and c-jun are involved in cellular growth and differentiation. They act as transcription factors and have been shown to regulate various genes [1,16,21]. Additionally, a role in the induction of programmed cell death or apoptosis is suggested by recent evidence [6,14,23,24]. This is an essential mechanism for cell clearance in the frame of developmental and pathological processes. One of its characteristics is cleavage of DNA by activation of endonucleases. The free 3′OH ends of the resulting DNA fragments can be visualized by in situ nick-end labeling [6,11]. With this method, we found labeling of endothelial cells and myocytes following cardioplegic arrest and reperfusion. For several reasons, a quantification of cells undergoing apoptosis and a comparison of different cardioplegia groups appeared difficult. The marked cells were unevenly distributed with the highest concentration of labeled myocytes in the subendocardial layer. The thickness of the atrial wall varied in different samples. Additionally, distinct differences between patients receiving the same cardioplegic solution were observed. Due to the rapid clearance of apoptotic cells only a part can be visualized and a considerable amount of cells may be lost with only few apoptotic cells being visible at a certain point of time [6]. Therefore, we consider our data on in situ nick-end labeling rather in a qualitative than in a quantitative way. However, all prebypass samples were virtually clear of apoptotic cells. A apoptosis was observed in rabbit hearts following ischemia and reperfusion [12]. Cardiac myocytes of dogs with chronic heart failure displayed apoptosis [22], which in fact has already been suggested as a basic mechanism for development of myocardial insufficiency in man [4]. Finally, apoptotic cells have been found in diseased sinus nodes excised for long QT syndrome [17]. These findings support a role of apoptosis in reperfusion damage and in cardiac pathophysiology. Further studies are clearly warranted to evaluate the impact of programmed cell death.

Our study suggests an induction of early immediate genes following cardioplegic arrest and reperfusion in human hearts. With in situ nick-end labeling we observed apoptosis in myocardial tissue of patients undergoing CABG. Increasing evidence supports an important role of early immediate genes in cellular responses to stress. A deleterious consequence of protooncogene induction may be programmed cell death. Therefore, molecular biology may offer additional tools for investigating cardiac pathophysiology, in particular in respect to cardiac surgery and evaluation of different cardioplegic strategies.
Fig. 4. In situ nick-end labeling of DNA fragments in biopsies of the same patient before (panel a, 250 × ) and after cardioplegic arrest and reperfusion (panel b, 500 × ). For better visualization of negative nuclei sections were counterstained with methyl green.

References


Appendix A. Conference discussion

Dr A. Mazzucco (Verona, Italy): Thank you Dr Aebert. This is clearly a very stimulating suggestion for people who are trying to protect the hearts during the surgical manipulation, and it is really putting under some different perspective all the options between operations without cross-clamping of the aorta, without ischemia, blood cardioplegia, crystallloid cardioplegia. So with this in mind, have you considered doing the same experiments under the experimental condition of a blood cardioplegia?

Dr H. Aebert: We agree that it would be interesting to perform the same analysis in patients receiving blood cardioplegia and we are actually preparing such a study.

Dr A. Mazzucco: Could you have an idea of which proportion of preconditioning factors and oncogenes are stimulated by the stress induced on the heart? I mean, can you say are there different situations in which the favourable effects can balance the deleterious effect of the stress?

Dr H. Aebert: With respect to preconditioning, species differences have to be considered. Ischemic preconditioning has been demonstrated in dog and rat hearts, in other species like in rabbits there are contradictory data from various groups. Yesterday in the poster session, Dr Cremer from Kiel presented clinical findings indicating that there was no preconditioning in human hearts following two periods of 5 min aortic cross-clamping before cardioplegic arrest. Another group in this session presented evidence for some mid-term attenuation of troponin T release after ischemic preconditioning in man. I think that at this point of time it is too early to build up a balance between potentially protective and potentially deleterious effects of stress or early immediate gene induction in the complex situation of patients undergoing open heart surgery.

Dr J. Vaage (Stockholm, Sweden): You said that you took a sample during reperfusion. How long after declamping was that sample taken?

Dr H. Aebert: That was after the end of cardiopulmonary bypass. The mean reperfusion time in the groups was about 35 min.

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Dr J. Vaage: Did you try to correlate the time of cross clamping with the gene expression?

Dr H. Aebert: We did this in isolated perfused rat hearts. After 20 min of cardioplegic arrest followed by reperfusion there was a significant induction of various early immediate gene mRNAs. Vice versa, we found a significant induction after cardioplegic arrest followed by 20 min of reperfusion. The mRNA levels showed a further increase with longer cardioplegia and reperfusion times, respectively.

Dr H. Huysmans (Leiden, The Netherlands): I would like to ask you whether you are quite sure that you are only measuring the effect, or lack of effect, of cardioplegia, or that other factors might have been involved, like mechanical damage to the heart tissue?

Dr H. Aebert: This is a good point. We cannot exclude that some other form of stress, like mechanical stress, may have been involved in induction of these genes. However, the differences between groups receiving different cardioplegic solutions makes it unlikely that this was the decisive factor. Aditionally, we found different patterns with different cardioplegic solutions in isolated unloaded rat hearts where we can better exclude the possible effects of mechanical stress.