Hypoxia induces heat shock protein expression in human coronary artery bypass grafts

Angelika Hammerer-Lercher a, *, Johannes Mair b, Johannes Bonatti c, Stefan B.C. Watzka c, Bernd Puschendorf a, Stephan Dirnhofer d

a Department of Med. Chemistry and Biochemistry, Division of Clinical Biochemistry, University of Innsbruck, Fritz-Pregl-Strasse 3, A-6020 Innsbruck, Austria
b Department of Cardiology, University of Innsbruck, Innsbruck, Austria
c Department of Cardiac Surgery, University of Innsbruck, Innsbruck, Austria
d Department of Pathology, University of Innsbruck, Innsbruck, Austria

Received 7 September 2000; accepted 19 December 2000

Abstract

Objective: Heat shock proteins (HSPs) are molecular chaperones which are essential for cell survival. Heat shock and hypoxia markedly increase the expression of several HSPs in various tissues, i.e. heart. In our in vitro study, we investigated whether HSPs are inducible in human vessels which are used as coronary artery bypass grafts. Methods: We used remnants of the saphenous vein and the internal mammary artery from 34 patients undergoing coronary artery bypass surgery. Each vessel was divided into segments, one for control conditions at 37°C (5% CO₂–95% air), the remaining ones for thermal (30 min at 42°C) or hypoxic treatment (6 h oxygen deprivation with nitrogen). The expression of Hsp60, Hsp72 and Hsp73 was investigated by immunohistochemistry and Western-blot analysis. Results: Compared to controls, segments of the saphenous vein undergoing heat treatment showed significantly increased expression of Hsp72 in the intima (P=0.035) and Hsp73 in the media (P=0.003). In the internal mammary artery, Hsp72 and Hsp73 were expressed in the intima at significantly higher levels (P=0.042 each). A 6 h oxygen deprivation with nitrogen resulted in elevated levels of Hsp60 (media: P=0.048), of Hsp72 (intima: P<0.001 and media: P=0.004) and of Hsp73 (intima: P=0.029) in the saphenous vein. In the internal mammary artery, Hsp73 expression was significantly enhanced (intima: P=0.048 and media: P=0.017). The results were confirmed by Western-blot analysis in representative veins. Conclusions: These findings demonstrate the common cellular defense mechanism of HSP expression in response to stress in coronary artery bypass grafts. Hypoxia and heat treatment strongly induce Hsp72 and Hsp73 expression in human coronary artery bypass grafts. © 2001 Published by Elsevier Science B.V.

Keywords: Arteries; Cardiovascular surgery; Hypoxia/anoxia; Preconditioning; Veins

1. Introduction

Pathophysiological stresses such as ischemia, oxidative stress, amino acid analogues, heavy metals, and heat shock are known to increase the expression of genes encoding stress, or so-called heat shock proteins (HSPs). During such cellular injuries, HSPs are responsible for repair or degradation of denatured proteins [1–4]. These cytoprotective proteins maintain protein conformation and cellular homeostasis, thus enhancing the cells ability to survive metabolic or oxidative stress. In the absence of stress, many HSPs act as molecular chaperones, which play a vital role in normal cellular processes. They facilitate folding, assembly and disassembly, as well as translocation of other proteins. There are different families of HSPs classified by their molecular masses in kilodalton (kDa). One of the best characterized is the Hsp72 kDa protein of the Hsp70 family which has been associated with cellular protection.

Overexpression of HSPs is considered an important

*Corresponding author. Tel.: +43-512-507-3522; fax: +43-512-507-2876.
E-mail address: angelika.lercher@uibk.ac.at (A. Hammerer-Lercher).

Time for primary review 25 days.
means of cell protection during physiological stress [3–8]. In Hsp72 rich cells, protein aggregation and death due to lack of ATP is suppressed, and in heart tissue the zone of myocardial necrosis is diminished. For example, transgenic mice expressing Hsp72 at high amounts showed reduced sizes of myocardial infarction after ischemia and reperfusion compared to non-transgenic mice, which is evidently due to the beneficial effect of excess Hsp72 [9,10]. In similar experiments, Trost et al. [11] reported myocardial dysfunction but no myocardial infarction after short ischemic periods. Moreover, a correlation was found between the degree of HSP induction and the level of myocardial protection [12].

Human endothelial cells have been reported to express HSPs after thermal stress [13]. We investigated HSP expression in human vessels used as coronary artery bypass grafts. These vessels are exposed to a variety of stress factors such as mechanical trauma and hypoxic injury during surgery and degeneration during the postoperative period [14,15]. As HSPs are essentially involved in protection from cellular damage, the aim of our study was to analyze their HSP expression pattern in response to heat shock and hypoxic stress in human coronary artery bypass grafts.

2. Methods

2.1. Patients

From thirty-four patients (four females and thirty males; average age 64±13 years) undergoing coronary artery bypass grafting remnants of the internal mammary artery (IMA) and saphenous vein (SV) were obtained. The study conforms with the principles outlined in the Declaration of Helsinki and was approved by the local ethical committee. All patients gave their written informed consent. Eighteen patients suffered from hypercholesterolemia, nine patients from diabetes and nineteen patients had hypertension. Seven patients were smokers. All patients had three vessel coronary artery disease. Presurgery medication were acetylsalicylic acid in twenty-nine patients, angiotensin-converting-enzyme inhibitors in seventeen patients, allopurinol in six patients, β-blockers in eighteen patients, nitrates in twenty patients, statines in twenty-four patients, diuretics in nine patients and calcium channel blockers in fifteen patients.

2.2. Anaesthesia

Anaesthesia was induced with midazolam and fentanyl in all patients. Endotracheal intubation was facilitated with vecuronium. Anaesthesia was maintained using a continuous infusion of fentanyl and midazolam. Additional bolus doses of fentanyl and isoflurane were administered according to clinical requirements. All patients received an initial bolus dose of aprotinin over a period of 15 min followed by a continuous infusion. Standard cardiopulmonary bypass technique (roller pump, membrane oxygenator and cardiotomy reservoir) with moderate systemic hypothermia (core temperature 30–32°C), topical cooling of the heart, and aortic crossclamping was used in all patients. Myocardial protection during aortic crossclamping was achieved by cold, multiple-dose hyperkalemic cardioplegia (St. Thomas’ Hospital II solution).

2.3. Tissue specimens

The saphenous vein was dissected for bypass grafting using standard surgical technique. Dilation of the vein used for grafting is a standard procedure to check leakage of side-branches and to overcome spasm. To obtain similar conditions for the vein-segment used for our investigations compared with the venous graft, all segments were dilated with an equal pressure of 200 mmHg. According to Bonchek et al. [16], this pressure does not affect the endothelium, and veins are functionally indistinguishable from nondistended ones. The internal mammary artery was dissected on a pedicle from the left thoracic wall. Immediately after excision, the segments of vessels for investigation were transferred to the laboratory at room temperature in HEPES-buffered RPMI 1640 culture medium (Sigma Aldrich, UK), supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml), gentamycin (25 µg/ml), amphotericin B (2.5 µg/ml), Glutamax™ 1 (2 mmol/l, all from Life Technologies, Paisley, UK), and sodium heparin (4 units/ml, Immuno, Vienna, Austria).

2.4. Organ culture

Vessel remnants were prepared under a laminar flow bench to provide sterile conditions for the organ culture. Adherent tissue was removed and the vessels were opened longitudinally to expose the endothelium. They were then cut into 2- to 6-mm stripes. The stripes were pinned with the endothelial face up onto a mersilene gauze resting on a silicon plate in a 50-mm sterile Petri dish (Bibby Sterilin, Stone, Staffs, UK). The segments were incubated at 37°C, 5% CO₂–95% air in RPMI 1640 culture medium (Sigma Aldrich) containing sodium bicarbonate (23.81 mmol/l) in place of HEPES and 30% fetal calf serum (Sigma Aldrich) and antibiotics as described above. Vessels were kept in culture for 21±5 h before starting the experiment to allow recovery from injuries caused by dissection, transport, and preparation for organ culture. From each vessel, one stripe served as a control, and the experiments were done with the remaining stripes of the same vessel.

2.5. Heat shock

The vessels (IMA: n=5, SV: n=5) were either heat shock treated for 30 min at 42°C in HEPES–RPMI 1640,
or subsequently additionally washed with medium and further incubated at 37°C with RPMI 1640 culture medium for 6 h (IMA: \( n=10 \), SV: \( n=12 \)) to receive optimal heat shock response for Western blot analysis and immunohistochemistry. The control vessels were treated the same way apart from remaining at 37°C and 5% \( \text{CO}_2 \)-\% air. For immunohistochemistry, the cultured vein and artery segments were fixed in 3.7% formalin (pH 7.4) overnight, processed, and then embedded in paraffin. For Western blot analysis, the segments were shock frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\) until analysis.

2.6. Hypoxia

Vein and artery segments (IMA: \( n=15 \), SV: \( n=15 \)) were washed with HEPES buffer (pH 7.4) containing HEPES (10 mmol/l), NaCl (145 mmol/l), KCl (5 mmol/l), MgCl\(_2\) (1 mmol/l) and CaCl\(_2\) (2.5 mmol/l). Then they were exposed for 6 h to hypoxic stress via oxygen deprivation with nitrogen. For achieving hypoxia we used a self constructed gas-tight chamber which was built according to Siegmund et al. [17]. This chamber was placed into the incubator and could be gassed with humidified nitrogen for a desired period of time to obtain hypoxic conditions. For the hypoxic period, the organ cultures were kept in HEPES buffer (pH 7.4) without glucose. Controls remained for this period of 6 h in RPMI 1640 culture medium in a standard incubator (5% \( \text{CO}_2 \)-\% air). Processing for Western blot analysis and immunohistochemistry were performed as described above.

2.7. Immunohistochemistry

Immunohistochemistry was carried out after deparaffination and rehydration of slides using specific monoclonal antibodies against Hsp60 (SPA 806, dilution 1:500), Hsp72 (SPA 810, dilution 1:200), Hsp73 (SPA 815, dilution 1:300, Stressgene, Victoria, Canada), and factor VIII (von Willebrand factor, A 0082, Dako, Copenhagen, Denmark) after scrupulous testing of both antibody dilution and antigen retrieval methods. Biotinylated secondary reagents and a streptavidin–biotin complex (P 0397 Dako) together with DAB-development (Sigma, Munich, Germany) were applied for visualization of the immune reaction. Antigen retrieval was accomplished by wet autoclavation (10 min, 1 bar) of the sections for HSPs in citrate buffer (0.5 mmol/l, pH 6) [18]. Sections of a gastric cancer with known strong HSP expression from a 75-year-old male patient served as positive controls for antibody staining in each immunohistochemic run.

The immunostained sections were evaluated independently by light microscopy by two of us (A.L. and S.D.). The distribution of intracellular localisation of HSPs was assessed by a 1000X high-power field magnification using oil immersion on an Olympus BX50 microscope (data not shown). Thus, each case was reviewed twice by each investigator and interobserver and intraobserver agreement was evaluated. Intraobserver-variability was <3%, and interobserver-variability was <5%. Interobserver disagreement was discussed and settled by means of a double-head microscope. A complete lack of HSP-positive cells was scored as 0, scattered HSP-positive cells (up to 20%) as 1, and strong HSP positivity as 2.

2.8. Immunoblotting (Western blot)

Segments were extracted in liquid nitrogen and immersed for 10 min in 62.5 mmol/l Tris–HCl and 50 mmol/l dithiothreitol (DTT) supplemented with 0.1% sodium dodecyl sulfate (SDS) and 4% Complete™ (protease inhibitor cocktail tablets, cat. no. 1697498, Boehringer Mannheim, Germany) and centrifuged for 10 min at 4°C and 1000 g. The supernatant was taken for immunoblotting.

Protein concentrations were determined by the Bradford dye-binding procedure of Bio-Rad (500-0006 Bio-Rad, Germany). Equal amounts of total protein were loaded onto 7.5% ready-to-use SDS-Minigels (161-0900 Bio-Rad, Germany). Equal amounts of total protein were loaded onto 7.5% ready-to-use SDS-Minigels (161-0900 Bio-Rad) and separated at 200 V and 15°C. Recombinant human Hsp60 (SPP-740), Hsp72 (SPP-755) and Hsp73 (SPP-750, Stressgene) were taken as positive controls. Proteins were then transferred onto nitrocellulose and an equal transfer was confirmed visually by Ponceau-S solution staining (1142372 Boehringer Mannheim) which was washed off afterwards. Nitrocellulose was incubated for 48 h at 4°C with 7% bovine serum albumin blocking solution (pH 7.4), washed with 0.1% Tween 20 (822184, Merck, Darmstadt, Germany) in phosphate buffered saline (PBS, pH 7.4) and then incubated with monoclonal antibodies specific for Hsp60 (SPA-806 at 1:6000 dilution) and Hsp73 (SPA-815 at 1:10 000 dilution) for 45 min at room temperature, and Hsp72 (SPA-810 at 1:10 000 dilution, Stressgene) overnight at 4°C. The nitrocellulose was washed with 0.1% Tween and further incubated with secondary antibodies anti-mouse Ig (NA 931 at 1:3000 dilution) for 20 min and anti-rat Ig (NA 932 at 1:9000 dilution) for 60 min at room temperature (NA 931/NA 932: horseradish peroxidase-linked whole antibody from sheep, Amersham Life Science, UK) and washed with 0.3% Tween. ECL™-detection (electrochemiluminescence, RPN 2106, Amersham Life Science) with documentation on X-ray films was used for detection of the proteins. Afterwards, the bands on X-ray films were evaluated visually as well as by scanning with a densitometer (E·script 440, ATH Analysentechnik Hirschmann, Taufkirchen, Germany). The 2D-evaluation program was used to compare the background corrected total absorbance of the different bands representing HSPs.

2.9. Statistics

Marginal homogeneity test of STATXACT 3 software (Cytel Statistical Software, Cambridge, MA, USA) was
Fig. 1. Hematoxylin and eosin staining: (A) Hematoxylin and eosin staining showed no tissue damage during organ culture. Lumen of the cultured saphenous vein at the top (×200). (B) Factor VIII staining was used as positive control for antigen integrity and proved presence of an intact endothelium. Lumen of the cultured artery on the left side (×400). The arrow points to the endothelium. Abbreviations: I, intima; M, media.
used to determine the significance between unstressed and stressed vessels (with heat shock or hypoxia). Results were considered significant in case of $P<0.05$.

3. Results

3.1. Organ culture

Hematoxylin and eosin staining of the paraffin embedded segments confirmed an intact layer of endothelium in all vessels and revealed no damage due to organ culture up to 7 days (Fig. 1A). Factor VIII staining served as positive control for antigen integrity and, moreover, proved the presence of an intact endothelium overlying the normal vessel wall (Fig. 1B).

3.2. HSP expression in response to heat shock

Immediately after a 30-min lasting heat shock, the expression of HSPs was rather low (data not shown) and no clear differences between stressed and unstressed vessels could be found. Expression was higher after subsequent 6 h of recovery in stressed compared to unstressed vessels. A 30-min period of heat shock at 42°C and 6 h recovery resulted in a markedly enhanced expression of Hsp72 and Hsp73 in the IMA and the SV. Immunohistochemically, the SV showed a significantly higher expression of Hsp72 in the intima ($P=0.035$) and of Hsp73 expression in the media ($P=0.003$; data not shown). Western blot analysis quantified by densitometric scanning of an X-ray film of a representative SV tissue specimen showed a 7.5-fold increase of Hsp72 (Fig. 2A) and a 2.3-fold increase of Hsp73 (Fig. 2B) expression compared with controls. In the IMA, Hsp72 and Hsp73 (Fig. 3) were both expressed in the intima at significantly higher rates ($P=0.042$ each). Moreover, immunohistochemistry and immunoblotting revealed basal expression of Hsp72 and Hsp73 in control vessels of saphenous vein (controls of Fig. 2) and internal mammary artery specimens. Immunohistochemically, expression of Hsp60 revealed no significant differences between stressed and unstressed IMA and SV.

3.3. HSP expression in response to hypoxia

Application of hypoxic stress for 6 h resulted in a marked induction of HSPs in the coronary artery bypass grafts. Immunohistochemistry of the saphenous vein showed a significant increase of Hsp72 (Fig. 4) in the intima ($P<0.001$) and the media ($P=0.004$). Densitometric scanning of the X-ray film of three investigated saphenous veins showed an average 5-fold increase of Hsp72 in the stressed SV compared to the unstressed SV, demonstrating a significant induction of Hsp72 after hypoxia (Fig. 5A). The immunohistochemical expression of Hsp73 in the intima ($P=0.029$) of the SV was also significantly higher. The above-mentioned three representative veins were also tested for Hsp73 expression by Western blot analysis (Fig. 5B). Densitometric scanning of these specimens confirmed this higher expression of Hsp73 compared with controls. Fig. 6 shows the distribution of each score in per cent of the total number of investigated IMA ($n=15$) or SV ($n=15$). The internal mammary artery exhibited a significant increase of Hsp73 in the intima ($P=0.048$) and a highly significant increase in the media ($P=0.017$, Fig. 4C and D). Immunohistochemically, the Hsp60 response of the segments was rather weak, and only in the SV we found a trend of enhanced induction of Hsp60 expression in both layers, intima ($P=0.051$) and media ($P=0.048$). Fig. 6 points out the differences of
Fig. 3. Immunostaining of heat shock treated vessel segments without recovery: in the IMA, a 30-min exposure to 42°C and a subsequent recovery period of 6 h, resulted in a significantly higher increase of Hsp72 in the intima (B, $P=0.042$) compared to control (A). Lumen of the vessels at the top ($\times400$).
Fig. 4. Immunostaining of hypoxic treated vessel segments: there was a basal expression of Hsp72 (A, ×400) and of Hsp73 (C, ×400) in the segments of unstressed vessels. The SV revealed a significantly higher expression of Hsp72 in the intima (B, ×400, P<0.001) and the media (P=0.004) compared to control (A). Lumen of the SV at the top. The IMA showed significantly higher levels of Hsp73 expression in the media (D, ×400, P=0.017) compared to control (C). Lumen of the IMA at the top.

Hsp60, Hsp72 and Hsp73 expression between stressed and unstressed SV, and Fig. 6B the difference of Hsp73 expression between stressed and unstressed IMA.

Immunohistochemistry and immunoblotting showed basal expression of Hsp72 and Hsp73 in control vessels of SV (controls of Fig. 5; Fig. 4A) and IMA (Fig. 4C). Furthermore, after heat shock, heat shock and a 6-h recovery period or after hypoxia, Hsp72 and Hsp73 were mainly localized in the nucleus and the cytoplasm, while Hsp60 after hypoxia was mainly found in the cytoplasm. We could not find any differences in the HSP distribution pattern after stress from the patterns of the corresponding control vessels.

4. Discussion

Previous studies have documented that in response to various physiological stresses, such as ischemia, oxidative stress, or heat shock, many mammalian cells show an increased synthesis of heat shock proteins [13,19]. We used heat shock of 42°C, a well-established inducer of HSP expression, to test whether HSP expression is in principal inducible in human coronary artery bypass grafts in vitro. Immediately after 30 min of heat shock exposure, HSP levels were low and there were no clear differences in the expression of HSPs between stressed and unstressed vessels, but after the subsequent 6 h culture, HSP expression was significantly higher in stressed vessels compared with controls. Moreover, Udelsman et al. [20] found maximum HSP expression 3–12 h after heat stress application in the aorta of Wistar rats. By immunohistochemistry, we found a pronounced increase of Hsp72 and Hsp73 in the SV, which was unequivocally confirmed by Western blot analysis. We also showed significantly higher increases of Hsp72 and Hsp73 in the IMA by immunohistochemistry. Hsp60 could not be found at significantly higher levels after heat treatment in both, IMA and SV, and basal staining of Hsp60 was low. In accordance with these results, it has been reported that Hsp60 expression...
occurred in endothelial cells of large human vessels, but not of small vessels, such as IMA and SV [21]. Expression of Hsp60, due to strong hemodynamic forces in large vessels, was moreover suggested to induce an initiating inflammatory process in atherosclerosis which rarely occurs in small vessels.

Similarly to our results, Welch et al. [19,22] detected Hsp72 even in unstressed human cell-lines. They described high levels of HSPs in some unstressed human cell-lines, indicating their involvement in various cellular processes under unstressed conditions. Hsp72 is present in some unstressed primate cells as well [23]. In our organ culture system, we observed basal levels in the intima and media of the coronary artery bypass grafts. This confirms the results of Welch et al. [19,22] on the level of an organ culture system. However, a possible cause for basal levels of HSPs could be the surgical stress itself. To allow recovery from surgical manipulation, the segments were cultured under optimized conditions until the next day, before using them for the experimental procedures.
According to Amberger et al. [24], after this 24-h period, stress induced Hsp60 expression is supposed to be back at basal levels, but protein levels of Hsp72 can still be high. However, the new synthesis of Hsp72 mRNA is almost completely suppressed 24 h after heat shock [25].

Furthermore, Welch et al. [19,22] investigated the distribution of HSPs in rat embryo fibroblasts, normal rat kidneys, and baby hamster kidneys. After a 3-h heat shock treatment, Hsp72 was mainly localized within the nucleus and nucleolus, but after an 8 h recovery period, most of the Hsp72 was in the cytoplasm. This colocalization with the nucleolus is suggested to be due to the repair for normal nucleolar function, and the distribution within the cytoplasm may be responsible for the proper function of the translational machinery of the ribosomes [19,22,26]. In our experiments, we observed intensive staining for Hsp73 and for Hsp72 in the nucleus and the cytoplasm of the cells, whereas Hsp60 was localized in the cytoplasm.

Various studies on rat and mouse models demonstrate the protective effects of HSPs against subsequent injury. Loktionova and Kabakov [27] suggested that heat-inducible HSPs can protect ischemia-stressed cells, e.g. during surgical operation, by preventing the ATP loss provoked protein dephosphorylation and breakdown of the actin cytoskeleton. In a study with Fischer rats, hypertension was reported to induce Hsp70 in the aorta, which suggested that Hsp70 may play a role in protecting the vasculature from damage during hemodynamic stress [28]. Nomura et al. [29] reported an improved recovery of ventricular function as well as endothelial function after hypothermic cardioplegic ischemia in an immature lamb heart model preconditioned with heat treatment. A whole-body hyperthermia at 42°C for 15 min of New Zealand white rabbits, followed by a 24-h later onset of myocardial ischemia for 30 min, and 3 h of reperfusion resulted in a diminished size of myocardial infarction [6]. Similar results have been obtained by Marber et al. and Donnelly et al. [7,8]. In addition, a study with Tru-cut biopsies of the myocardium from patients undergoing cardiac surgery showed an increase of Hsp72/73 after brief alternating periods of normothermic ischemia and reperfusion [30]. Therefore, we tested the HSP expression in response to hypoxia in human vessels.

After optimization of our experimental conditions, a 6-h period of hypoxia was used to test the HSP response of coronary artery bypass grafts. The HSP expression following hypoxia compared with controls was stronger in veins than in artery segments. This finding might be due to the different biological properties of the vessels. The IMA consists of several layers of elastic lamina and only a few muscle cells. The higher amount of elastin in the IMA may render this type of vessels more resistant to ischemia, because elastin is a relatively inert tissue and has a low metabolic rate [31]. Furthermore, in contrast to the inert SV, the IMA contains vasoactive properties and shows a low incidence of atherosclerosis [32]. This may also render the IMA more stress-insensitive than the SV. Additionally, in long-term survival studies [33–36] the IMA graft revealed a superior outcome (superior survival, intervention-free survival and event-free survival) compared to the SV. Therefore, the capability of SV grafts to express cytoprotective HSP may be of benefit and the further induction of these proteins could improve the outcome of SV grafts. This latter hypothesis will have to be proved in further studies.

In summary, to the best of our knowledge this is the first study on HSP expression in human coronary artery bypass grafts. Our results demonstrate the potent cellular defense mechanism of HSP expression in response to stress, such as hypoxia and heat, in coronary artery bypass grafts. In addition, basal expression of Hsp72 and Hsp73 was found in the SV and the IMA. After hypoxic stress, saphenous vein segments showed an enhanced expression of Hsp72 in the intima and media, and higher levels of Hsp73 in the intima compared to controls. In the IMA, there was a significant increase of Hsp73 in the intima and media. Furthermore, there was increased Hsp60 expression mainly of the SV after hypoxia, but not after heat shock. Because of the established cytoprotective effects of HSPs, our findings may have implications regarding the resistance of coronary artery bypass grafts to pathophysiological stress. Particularly, the stronger HSP expression in the SV may be of significance for the protection from vascular damage due to the unphysiological hemodynamic stress [28] after grafting into the arterial circulation.

Acknowledgements

The excellent technical assistance of Sabine Jöbstl, Department of Pathology, University of Innsbruck, Austria is highly appreciated. The authors thank Professor K.P. Pfeiffer, Department of Biostatistic and Documentation, University of Innsbruck, Austria for assistance in the statistical analysis of our data.

References


