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ORIGINAL ARTICLE

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Polyclonal anti-thymocyte globulins influence apoptosis in reperfused tissues after ischaemia in a non-human primate model

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Abstract Reperfusion triggers the expression of inflammatory cytokines and adhesion molecules that increase the rate of apoptosis in the reperfused tissues after ischaemia, thus worsening the outcome of the grafts. Polyclonal anti-thymocyte globulins (pATGs) are able to reduce the number of lymphocytes as well as block adhesion molecules and induce apoptosis in T-lymphocytes through Fas-ligand. The aims of this study were to investigate the influence of pATGs on the prevention of apoptosis of reperfused tissues after ischaemia and to monitor their capability to enhance lymphocyte apoptosis thus decreasing the deleterious effects of ischaemia/reperfusion injury (IRI). Extremities of cynomolgus monkeys ($n=8$) were flushed via either the femoral or the brachial artery. After 60 min of ischaemia the limbs were reperfused with human blood. ATG was added to the blood in a therapeutic dose 20 min prior to reperfusion of the extremities. Surgically available limbs ($n=20$) were assigned to the following groups: ATG group ($n=10$) and control group (without ATG; $n=10$). DNA fragmentation analysis was performed in situ to detect apoptosis at the single-cell level. Our study shows an increased rate of muscle and connective tissue apoptosis in the control group compared with the ATG-treated group. Cells found in the vascular areas present different rates of apoptosis, with enhanced cellular death of endothelium and connective perivascular areas being observed in the control group. The group treated with ATG shows an increased rate of white blood cell (WBC) apoptosis in vascular and perivascular areas. Previous studies have shown that pATGs are able to induce apoptosis as well as complement-mediated cell death in peripheral T-lymphocytes in vitro. Our results confirm that pATGs not only increase the rate of apoptosis of WBCs in vivo but also have a protective effect on the reperfused tissue. This may alleviate the damage after reperfusion of solid-organ transplantation.

Keywords ATG · Ischaemia–reperfusion · Apoptosis · TUNEL · Morphology

Introduction

Ischaemia/reperfusion injury (IRI) in the early post-transplantation period is associated with an increased rate of acute rejection, delayed recovery of the graft function and late allograft failure leading to the loss of the graft [1, 2]. Central organ systems are particularly sensitive to IRI [3]. Cell death due to necrosis and apoptosis plays a key role in organ damage induced by IRI [4]. Ischaemia and the subsequent cessation of energy production due to the loss of oxygen supply and posterior reperfusion trigger the loss of osmotic

equilibrium and permeability of cellular membranes, leading to cell necrosis and failure of organ function. Although reperfusion is the only effective strategy to restore the tissue functionality, cells continue to die, partly because of the damage induced by ischaemia and partly due to the release of cytotoxic agents and the expression of adhesion molecules after reperfusion [5].

Apoptosis is a programmed and physiological death that can also be triggered by pathological conditions such as IRI. Several genes are involved in the enhancement of apoptosis after reperfusion; increased expression of the families of BCL-2 and p53 genes and higher levels of CD95 (Fas-ligand) are seen [6, 7, 8]. The investigation of IRI in vivo demonstrates that apoptotic cell death of the reperfused tissues is an important component of organ failure [9]. Damage induced during ischaemia, comprising hypoxia-related decrease of energetic metabolites, is capable of initiating the apoptosis cascade [10]. However, prolongation of ischaemia enhances the process of necrosis in the tissue. Restoration of the tissue supplies by reperfusion is important to allow the ischaemia-induced apoptotic-pathway to function. Thus, the tissue cells suffer apoptosis due to the influence of ischaemia and posterior reperfusion, which allows the necessary molecules to trigger and supply the apoptotic pathway. Furthermore, infiltrating leukocytes, as well as cytotoxic lymphocytes, enhance the deleterious effect of hypoxia and release of oxygen-free radicals, leading to tissue damage.

Polyclonal anti-thymocyte globulins (pATGs) are a group of potent immunosuppressive agents of broad spectrum that have been used in organ transplantation and haematological diseases since 1968. Although lymphocyte depletion constitutes the primary mechanism of the immunosuppressive effects of ATGs, other mechanisms, such as the blocking of adhesion molecules and apoptosis induction, are involved [11]. The aim of this study was to investigate whether polyclonal ATGs induce apoptosis of leukocytes in reperfused tissues after ischaemia in an in vivo non-human primate model and to ascertain whether this effect might reduce the rate of apoptosis of functional tissue cells.

Material and methods

Experimental procedure

The study was revised and approved by the Animal Care and Use Committee of the government of Bavaria under the registry AZ: 211-2531.33/00. The animals received human care in compliance with the *NIH Guide for Care and Use of Laboratory Animals* (NIH publication No.85-23, 1985). Eight cynomolgus monkeys with an average age of 52 ± 35 months and an average weight of 4.02 ± 0.85 kg were divided into two groups. The extremities of the monkeys were flushed under anaesthesia with 500 ml of Ringer's lactate solution at a temperature of 4°C, via either the brachial or the femoral artery. Blood was drained via the axillary or the femoral vein.

After 60 minutes of ischaemia the limb was reperfused for 1 h with oxygenated human blood of the corresponding blood group (BG), which was, in all cases, BG 0. Perfusion was carried out in a re-circulatory manner by means of a perfusion system [12]. Every surgically available limb was perfused ($n = 20$) and considered as a single experiment. Human blood was taken from different volunteers free from chronic or acute diseases 1 h prior to the beginning of reperfusion (500 ml). Both male and female donors were accepted. The freshly drawn blood was constantly held at 37°C, heparinised, and adjusted to a haematocrit of 30% by the addition of Krebs–Henseleit buffer. Blood from group 1 was free from ATG and served as control ($n = 10$). ATG (Fresenius, Munich, Germany) was added to the human blood in group 2 ($n = 10$), in a standard concentration of 1 mg/kg, 30 min prior to the start of reperfusion. ATG was diluted to 10 ml by means of phosphate-buffered saline solution before being added to the blood.

Histological and TUNEL analysis

Biopsy material was obtained from muscle and connective tissue from the limbs of the monkeys at the end of every experiment. Biopsies were stored in 4% formalin for 48 to 72 h and fixed in paraffin. Sections 6-μm thick were cut from the paraffin-embedded tissue, dehydrated and fixed in xylol for histological and in situ hybridisation studies. Haematoxylin–eosin staining was performed to assess the histological aspects of apoptosis related to IRI. DNA fragmentation in the reperfused tissues, as a marker for cell apoptosis, was analysed by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) [13]. In brief, after fixation, the samples were rehydrated and immersed in 3% H_2O_2 at room temperature to quench endogenous peroxidase activity. Residues of digoxigenin nucleotide were then added to the 3′-OH ends of double-stranded or single-stranded DNA by means of terminal deoxynucleotidyl transferase (TdT).

After incubation with standard anti-digoxigenin immunoperoxidase (Sigma, Hamburg, Germany), the sections were developed with diaminobenzidine (Dako, Glostrup, Denmark). Counterstaining with methyl green was performed to identify the cell type in serial sections. Slides incubated without TdT and lymph-node sections were used as negative and positive controls, respectively.

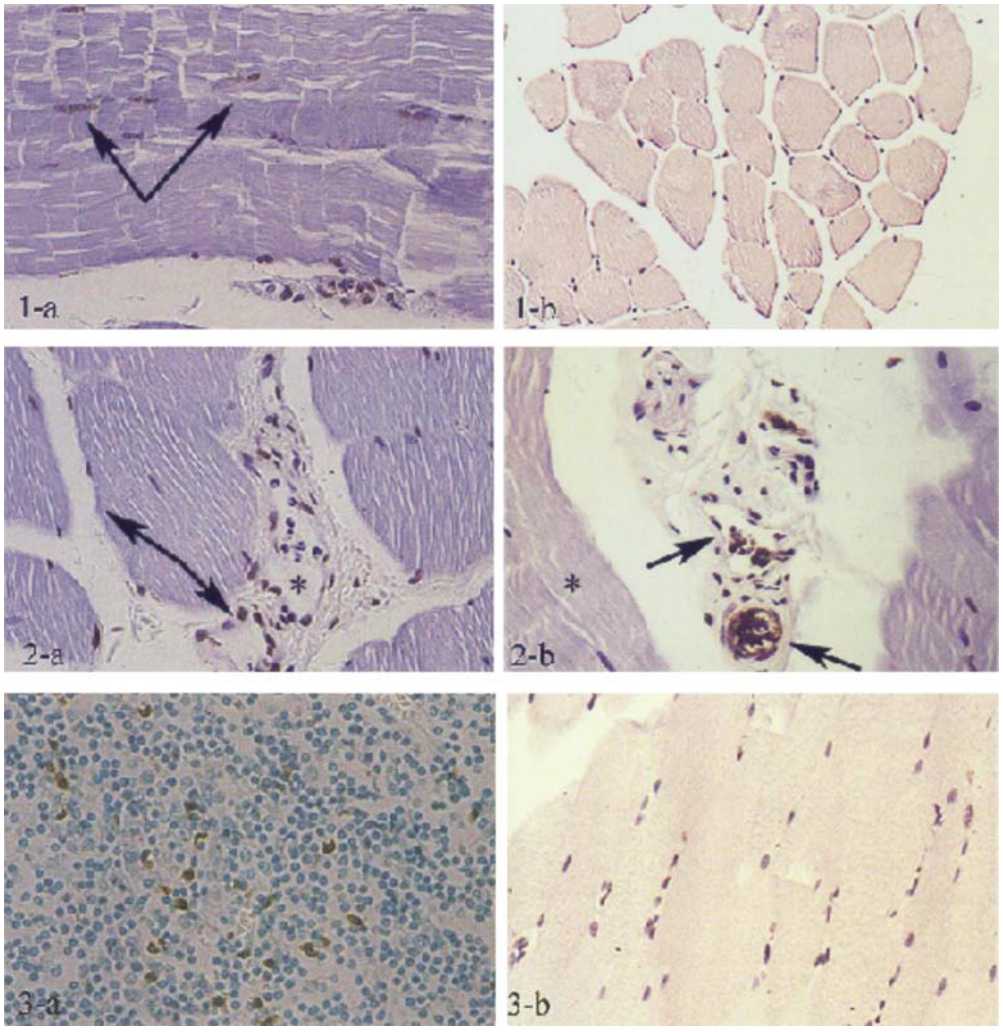

Fig. 1a,b Representative microphotographs of TUNEL-stained sections of reperfused muscle. **a** Apoptotic muscle nuclei are observed in this picture of the control group (*arrows*). **b** In the ATG group, the muscle structure is well conserved and the nuclei present no positive reaction when TUNEL stained. Both ×40

Fig. 2 a In the control group, positive TUNEL reaction is seen in muscle and endothelial cells (*arrows*). Non apoptotic WBCs are located in the lumina of the vessels and attached to the endothelial walls (*asterisk*). **b** Muscle nuclei presenting no positive TUNEL reaction in the ATG-treated group are shown (*asterisk*); There is a positive reaction in the WBCs present in the vessels (*arrows*). Both ×40

Fig. 3 a Positive control of the technique performed in a lymph-node section (counter-stained with methyl green) **b** Negative control of a muscle section after omission of a step of the TUNEL reaction. Both ×40

Those controls were performed to assess the quality of the technique and dismiss unspecific reactions.

Counting and statistical analysis

The criteria of apoptosis were morphologically established on the basis of cytomorphological signs, such as condensation of nuclear chromatin, and verified with a positive TUNEL reaction. The number of TUNEL-positive cells in muscular, connective and vascular structures was counted and divided by the total number of cells present in those structures per field, to determine the percentage of TUNEL-positive cells. Ten different fields for each section were analysed twice. A scale from 0 to 3+ was used to express the incidence of apoptotic cells and tissue damage in reperfused tissue in a semi-quantitative assay, this scale being correlated with the percentage of apoptotic cells. The counting was performed by two different observers, independently (A.B.-F. and R.G.). All values are expressed as mean ± standard deviation. Differences between rates were determined by McNemar's test. Values of $P < 0.05$ were considered to be statistically significant.

Results

Histological aspects of apoptosis were studied to verify whether IRI induces morphological changes characteristic of apoptosis, as well as to determine the extent of inflammation. Haematoxylin–eosin (H/E) staining of skeletal muscle showed increased inflammation and apoptosis-like nuclei in the control group in comparison with the ATG group. After H/E staining, the sections of skeletal muscle showed almost no histological features of inflammatory reaction and muscle degeneration after 60 min of ischaemia and posterior reperfusion in the groups treated with ATG.

Detection of apoptotic cells was by in situ labelling of DNA strand breaks. TUNEL staining clearly localised positive nuclei in reperfused muscle. The reaction product was brown, and minimal background was observed. These assays demonstrated that the incidence of TUNEL-positive nuclei was significantly ($P < 0.05$) reduced in the ATG-treated limbs compared with the control limbs after IRI. The percentage of apoptotic cells of the ATG group was decreased in skeletal muscle, connective tissue and endothelial cells in comparison with the control group, as shown in Fig. 1. In contrast, the apoptosis of white blood cells (WBCs) was significantly increased in the ATG-treated group in comparison with the control group ($P < 0.05$) (Fig. 2). Positive WBCs were identified by means of morphological characteristics under H/E staining and accepted as apoptotic after positive TUNEL reaction. Both percentages were statistically analysed in a contingency table, being the significant differences shown in Table 1. These results correlated well with the semi-quantitative assay performed. Positive and negative controls of the technique are shown (Fig. 3).

Discussion

Investigations of IRI in vivo have demonstrated that apoptotic cell death is an important component of organ injury [14]. Damages induced during ischaemia are capable of initiating apoptosis; however, restoration of metabolic processes during reperfusion seems to be essential to trigger the apoptotic pathway [13]. Induction of apoptosis in reperfused tissues after ischaemia has been related to the expression of several genes, basically the bcl-2 and CD95 (Fas-APO1) families. Further inflammation-related genes, such as the early responders c-fos and c-jun, have been hypothesised to take part in an adaptive reaction to tissue hypoxia, including cell apoptosis [15].

WBCs, including B-lymphocytes and T-lymphocytes and, especially, neutrophils, are closely related to the development of inflammatory reactions after ischaemia/reperfusion [16]. Cell-to-cell interactions with the endothelium, release of pro-inflammatory mediators and direct cytotoxicity are important mechanisms of organ and tissue damage [17, 18]. Furthermore, over-expression of Fas/FasL complex has been found on neutrophils, macrophages, T-cells, and vascular endothelial cells, after reperfusion, by means of immunohistochemical and mRNA analyses [19], suggesting the involvement of WBCs in triggering tissue apoptosis after reperfusion in a non-metabolic pathway. TUNEL is a reliable method for investigating the extent of apoptosis and has been extensively employed in transplantation and IRI models [20, 21].

pATGs are able to deplete and modulate peripheral blood T-cells. T-cell depletion involves active cell death, demonstrated by annexin V binding [22]. Maximal access and depletion occurred in blood, caused by complement-dependent lysis. However, a second mechanism of T-cell depletion would be an activation-associated apoptosis, independent from Fas and TNF-α [23, 24]. The aims of our study were to investigate whether pATGs would be capable of stimulating WBC apoptosis after reperfusion and to assess the possible prevention of apoptosis in the reperfused tissues. Our results show that pATGs are able to induce apoptosis of WBCs in vivo after ischaemia/reperfusion, in concordance with the results of Preville and colleagues, who showed a similar activity of pATGs in a transplantation model [11]. Furthermore, our results also show a decrease in the rate of apoptosis in reperfused tissues after ischaemia, suggesting a protective effect on IRI.

A similar protective effect in prevention of apoptosis of stem cells after treatment with pATGs was shown by Flynn and colleagues in an experimental study [25]. Those protective effects, which consisted of a reduction of the apoptosis rate of reperfused muscle, could be related to a direct effect of ATG on blocking the cell-to-cell interactions leading to cytotoxicity and cellular damage or, indirectly, to a reduction of the inflammatory mediators released after reperfusion, triggering the apoptotic pathways. Reduction of apoptosis is fundamental if the extent of inflammation after reperfusion injury is to be decreased [3]. Additionally, abrogation of early IRI-induced apoptosis might be a useful tool to prevent organ dysfunction after transplantation. The use of substances that prevent the early features of IRI,

Table 1 Percentages of TUNEL-positive cells. Differences between the ATG-treated and control groups are statistically significant for both WBCs and muscle cells (including endothelium and connective tissue). A semi-quantitative assay, showing presence of apoptosis and tissue damage in both groups, is presented

Group	White blood cells (%)		Muscle, connective and endothelial cells (%)		Semi-quantitative assay Apoptosis; tissue damage
	Apoptosis (+)	Apoptosis (−)	Apoptosis (+)	Apoptosis (−)	
ATG-treated ($n = 10$)	73.6 ± 6.7*	25.8 ± 7.1*	27.9 + 7.1*	72 + 7.2*	0/+
Control ($n = 10$)	22.6 ± 4.5	77.4 ± 4.4	33.9 + 4.6	65.9 + 4.7	+ + +

(*$P < 0.05$)

including pATGs, might decrease the rate of organ failure as well as provide a better outcome for the transplanted organs and for the patients.

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