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ORIGINAL ARTICLE

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Prevention of free-radical induced apoptosis by induction of human recombinant Cu, Zn-SOD in pig endothelial cells

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Abstract Vascular endothelial cells are the prime target in ischemia reperfusion injury. Growing evidence has shown that one of the main etiologies is considered to be reactive oxygen species (ROS) that induce endothelial-cell death either by necrosis or apoptosis. Cultured porcine endothelial cells were transfected with human copper, zinc-superoxide dismutase (h-Cu, Zn-SOD) to investigate whether these cells can prevent apoptosis from oxidative injury in vitro. The endothelial cells were cultured with SIN-1 (3-morpholinosydnonimine-N-ethylcarbanride) as a donor of peroxinitrite ($ONOO^-$). The control cells without the gene transfection developed characteristic apoptotic changes both morphologically and biochemically when they were incubated with SIN-1 of 200 M. However, the cells showed necrosis predominantly when the concentration of SIN-1 was 1,000 M. On the other hand, the cells transfected with h-Cu, Zn-SOD showed significantly less evidence of apoptotic change after exposure to SIN-1. Nitric oxide (NO) did not significantly affect the viability of either the control cells or the transfected cells. One of the potent ROS, peroxinitrite, is considered to play a significant role in ischemia reperfusion injury. SIN-1 can produce peroxinitrite in vitro that induces endothelial-cell damage by apoptosis. This type of cytotoxicity can be successfully prevented by transfection of the h-Cu, Zn-SOD into the cells.

Keywords Endothelium · Apoptosis · Reperfusion · Free radical

Introduction

Vascular endothelium is sensitive to injury from reactive oxygen species (ROS) [4, 14]. Indeed, such cytotoxicity is believed to be one of the main underlying etiologies in ischemia reperfusion injury of the endothelium after cold preservation for solid organ transplantation [6]. In this situation, the graft function is largely influenced by the viability of the vascular endothelium in the graft secondary to a production of ROS, such as O_2^-, H_2O_2 or NO [5]. Subsequent events include the development of a further burden of ROS as a result of the disintegration or activation of the endothelium, the liberation of various cytokines and vasoactive substances causing neutrophil infiltration and adhesion to the endothelial cells [26]. As a result, a second set of bursting oxygen radicals can develop from the activated macrophages and neutrophils; this impairs microcirculation and leads to lethal damage to the organ. The mechanism of the cytotoxicity is explained as direct damage to the cellular proteins, DNA and lipids by the ROS; this results in cell necrosis and/or apoptosis [22, 25].

Superoxide dismutase (SOD) is one of the first lines of defense against oxygen-derived free radicals, and it can prevent ROS-induced endothelial injury. The viable vascular endothelium contains copper, zinc (Cu, Zn)-SOD,

which is a dominant form of SOD [8]. In normal conditions, a steady state of balance is maintained between the production of ROS and their destruction by the cellular antioxidant. However, in pathological conditions such as ischemia reperfusion, SOD may not be able to cope with a rapid and overwhelming burden of ROS. This concept led us to conduct a novel approach, transfecting the human (h)-Cu, Zn-SOD gene into porcine aortic endothelial cells (PAEC) in vitro [21]. We found that the viability of such cells transfected with the h-Cu, Zn-SOD gene was significantly greater than that of non-transfected control cells when they were subjected to oxygen free radicals produced by a xanthine-hypoxanthine reaction. We conducted an in vitro study in order to investigate whether the PAEC transfected with h-Cu, Zn-SOD could prevent apoptosis from injury by ROS. For this purpose, we used S-nitroso-N-acetyl-DL-penicillamine (SNAP) as a donor for NO and 3-morpholynosidnomine-N-ethylcarbamide (SIN-1) as a donor for peroxinitrite ($ONOO^-$).

Materials and methods

Cells

In the animal experiments, the Principles and Regulations of the Animal Research Laboratory of the Nagoya University School of Medicine were followed. Porcine aortic endothelial cells (PAEC) harvested from the thoracic aorta of Landrace pigs were kept in culture. They were isolated, characterized, maintained in 10% fetal-calf serum (FCS) containing Dulbecco's modified eagles medium (D-MEM) and incubated at 37°C in 5% CO_2/95% air. The cells were morphologically evaluated by microscopy throughout the process of experimentation.

Reactive oxygen species (ROS)

SNAP and SIN-1 were purchased from Dojindo, Kumamoto, Japan. Peroxynitrite production via SIN-1 is based on the combination of its byproducts, O_2^- and NO, to form $ONOO^-$. As peroxynitrite induces oxidation of dihydrorhodamine to rhodamine, peroxinitrite production was confirmed by measuring the rhodamine concentration in the incubation media. Standard rhodamine and dihydrorhodamine were purchased from Sigma Chemical (St. Louis). Measurements of fluorescent intensity were made on a spectrophotometer (Shimazu RF-5,000, Shimazu, Tokyo) with excitation and emission wavelengths of 500 nm and 536 nm, respectively (emission slit widths of 3.0 mm). Peroxinitrite induced the oxidation of dihydrorhodamine to rhodamine in a linear fashion over the range of 0–1,000 μM (Fig. 1). The change in the ratio of rhodamine concentration was 0.6 ± 0.01 rhodamine per peroxinitrite.

Nuclear fragmentation and caspase inhibitor

The PAEC (1×10^6) with or without ROS were incubated for 12 h. They were collected by centrifugation and re-suspended in 300 μl of 10 mM Tris-HCl (pH: 7.5), 1 mM ethylene diamine tetra-acetic acid disodium salt (EDTA), 0.15 M NaCl, 1% sodium dodecyl sulfate (SDS), 0.2 mg/ml of proteinase K. After the PAEC was incubated overnight at 37°C, the DNA was extracted with phenol-chloroform, ethanol precipitated overnight, re-suspended in water and separated by electrophoresis in a 1%-agarose gel. The nuclei of the PAEC were morphologically evaluated by staining them with propidium iodide (PI) for evidence of nuclear fragmentation; they were examined under a confocal laser microscope (MRC-1024, Japan Bio-Rad Laboratories, Tokyo). For apoptosis inhibition assays, the cells were preincubated for 3 h with 200 μM of CPP32/Apopain inhibitor (Kamiya Biochemical, Seattle) prior to quantitative analysis by flow cytometer, EPICS Profile (Coulter, Hialeah, Florida).

Transfection of h-Cu, Zn-SOD

The details were described in our previous report [21]. Briefly, the fragment encoding recombinant h-Cu, Zn-SOD was excised and was inserted in the retroviral vector. Plasmid DNA was transfected into the retroviral packaging line, and the culture medium was harvested to infect amphotropic PA317 cells, which were then isolated into single colonies. The PAEC were infected using a producer line (PA317/SOD) with a high titer (1×10^4 colony forming units/ml). The antibiotic G418 was added on the following day, and the clones were isolated. Integration of h-Cu, Zn-SOD cDNA was confirmed by polymerase chain reaction (PCR).

Cytotoxic assay

The viability of the cells was tested with the use of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay by incubating the cells with ROS at their concentrations of 0, 200 and 1,000 μM. The PAEC without gene transfection were used as a control. The values were expressed as the percentile ratio to the cytotoxicity of the control cells, which were cultured in the medium free of ROS.

Quantitative assay of DNA fragmentation

DNA fragmentation was analyzed using a flow cytometric assay. The cells (2×10^5 cells/well) were plated in 6-well dishes in growth medium. After 24 h of incubation, they were washed two times and were incubated with or without ROS for 12 h at 37°C in 5% CO_2.

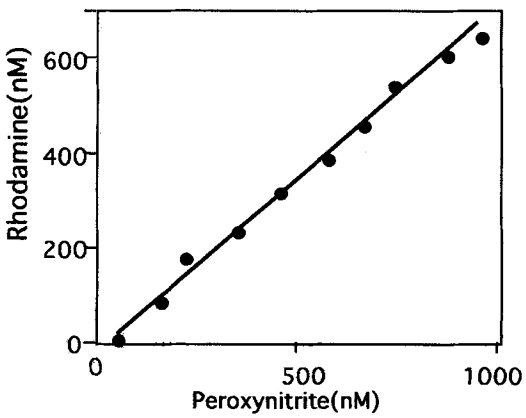

Fig. 1. Peroxinitrite-dependent oxidation of dihydrorhodamine: a linear increment of peroxinitrite concentrations against dihydrorhodamine is noted. Rhodamine concentrations were determined from fluorescent intensity measurement. The ratio of the change in the rhodamine concentration was 0.6–0.01 per peroxinitrite

At the end of the incubation period, they were lysed in a buffer with PI. Then the cells were analyzed for DNA contents by the flow cytometer.

Statistics

All data were expressed as the mean ± standard deviation (SD), and statistical analyses were performed using a one-way ANOVA. Statistical significance was defined as a P value below 0.05.

Results

Morphological findings

The control cells of the PAEC with the culture media only showed a typical cobblestone appearance (Fig. 2a). After exposure of the cells to SNAP, the cells did not significantly alter their morphology. However, with SIN-1 of 200 μM, it was characteristic that a significant number of the cells were shrunken and/or rounded with a bleb formation, indicating apoptosis (Fig. 2b). With SIN-1 of 1,000 μM, it was evident that some of the cells developed ballooning or disruption of the cell membrane with lysis of the nuclei, which was suggestive of necrosis (Fig. 2c). In contrast, the cells transfected with h-Cu, Zn-SOD showed less evidence of such changes after exposure to SIN-1 than did the cells without transfection (Fig. 2d,e,f).

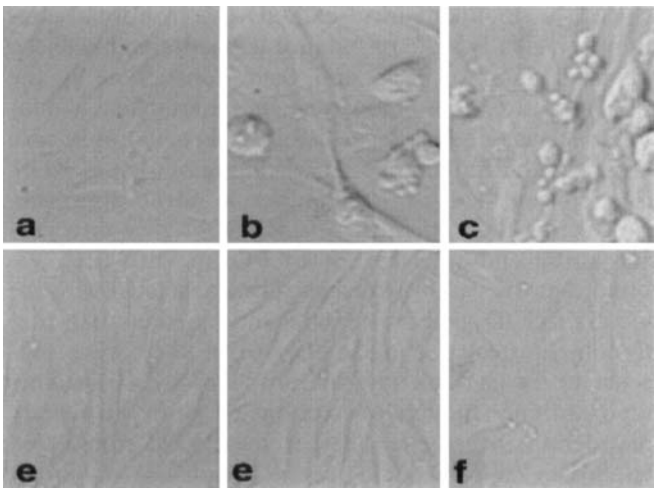

Fig. 2a–f. The effect of SIN-1 on the morphology of the porcine aortic endothelial cells (PAEC) and those transfected with h-Cu, Zn-SOD. Morphological changes were determined by using a confocal laser microscope showing: **a** the untreated control; **b** the control cells with SIN-1 of 200 μM; **c** the control cells with SIN-1 of 1,000 μM; **d** the untreated transfected cells; **e** the transfected cells with SIN-1 of 200 μM; **f** the transfected cells with SIN-1 of 1,000 μM

Nuclear fragmentation

The nuclei of the control cells cultured in vitro in the medium with SIN-1 of 200 μM were stained with PI in which the viable cells failed to show nuclear staining because of their intact cell membranes, but some of the cells showed faint staining of the nuclei with fragmentation. The nuclei of the cells with ballooning or with membrane lysis were clearly stained, and most of them were fragmented (Fig. 3). On the other hand, the cells transfected with h-Cu, Zn-SOD did not show a significant nuclear staining. Neither the control cells nor those with h-Cu, Zn-SOD showed a significant nuclear staining when the cells were cultured in the plain medium or in the medium with SNAP. The findings of the agarose gel electrophoresis were compatible with those of the morphological studies in which the control cells incubated with SIN-1 of 200 μM showed a typical multimer pattern characteristic of apoptosis, whereas the cells transfected with h-Cu, Zn-SOD did not develop a significant laddering. When the cells were incubated with SIN-1 of 1,000 μM, the control cells developed less laddering but did show a smear pattern indicative of necrosis. The cells with h-Cu, Zn-SOD developed no evidence of laddering, but there was slight evidence of a smear pattern when the concentration of SIN-1 in the medium was 1,000 μM (Fig. 4).

Cytotoxic assay

The viability of the control cells when they were incubated with SNAP in the concentrations of 200 and 1,000 μM were 97.9 ± 1.5% and 97.7 ± 1.5%, respectively, which did not significantly differ from those without SNAP (98.2 ± 1.5% and 98.2 ± 1.4%, respectively). When the control cells were incubated with SIN-1, the cytotoxicity was 78.5 ± 5.5% and 47.4 ± 9.0%, respectively. On the other hand, the cytotoxicity of the cells transfected with h-Cu, Zn-SOD was 98.6 ± 1.5 and 96.8 ± 1.5%, respectively, in the concentrations of 200 and 1,000 μM of SNAP, respectively. The cytotoxicity of the cells transfected with h-Cu, Zn-SOD was 88.5 ± 3.5% and 76.0 ± 4.0%, respectively, in the concentrations of 200 and 1,000 μM of SIN-1 (Fig. 5).

Quantitative assay of DNA fragmentation

Flow-cytometric analysis for quantification of the apoptotic cells that appeared in the sub-G1 peak showed that the DNA fragmentation was significantly suppressed for the cells pre-incubated with caspase-3 inhibitor (Fig. 6). The ratio of DNA fragmentation in the cells transfected with h-Cu, Zn-SOD was significantly

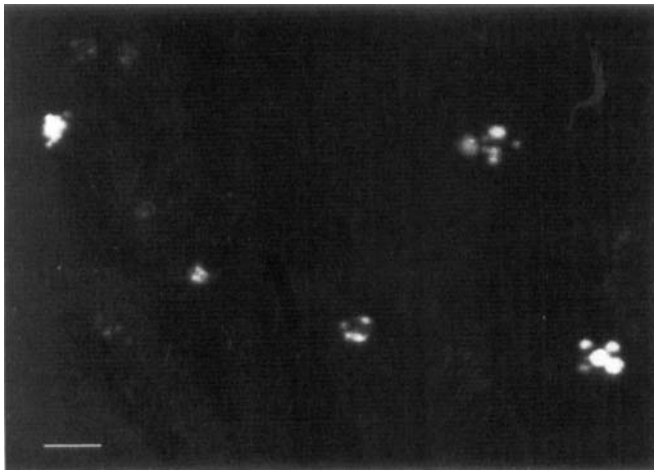

Fig. 3. Morphology of the untreated control porcine aortic endothelial cells. The cells were treated with SIN-1 (200 μM) for 12 h and were stained by PI (propidium iodide) and examined under the confocal laser microscopy (a *bar* denotes 10 μm)

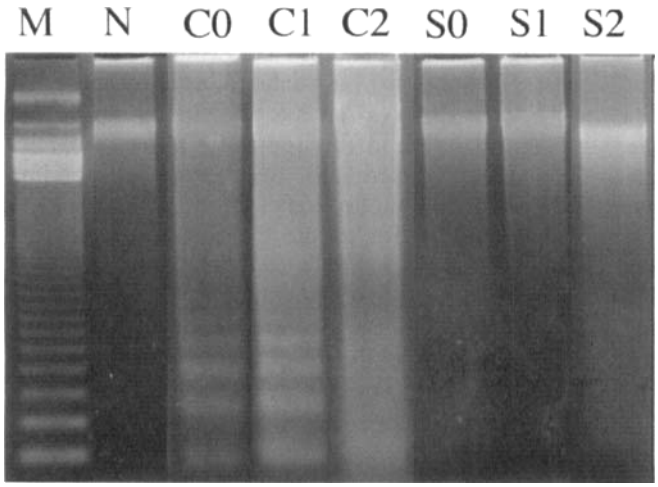

Fig. 4. Agalose gel electrophoresis of the endothelial cells. Each column of the electrophoretic pattern of the DNAs is designated as: *C0* the untreated control cells; *C1* the control cells with SIN-1 of 200 μM; *C2* the control cells with SIN-1 of 1,000 μM; *S0* the untreated transfected cells; *S1* the transfected cells with SIN-1of 200 μM; *S2* the transfected cells with SIN-1 of 1,000 μM. *M* marker, *N* negative control

less than that of the cells without transfection when they were incubated with SIN-1.

Discussion

Here we have shown that the endothelial cells develop apoptosis because of substances that produce reactive oxygen species (ROS), such as superoxide anion radical (O_2^-) and nitric oxide (NO). Confirmation of apoptosis was based on the findings by both morphological and biochemical studies. The superoxide anion radical is thought to participate with various pathological processes. Superoxide dismutase (SOD), for example, reduces endothelial injury by dismuting O_2^-. However, it is increasingly understood that this anion alone seems to have limited reactivity with most biological molecules [1]. To account for the apparent toxicity of O_2^-, recent observations have shown that NO, a product of the endothelial cell and a major form of endothelium-derived relaxing factor (EDRF), binds with O_2^- to form a far more reactive peroxinitrite ($ONOO^-$) [23]. Another possible mechanism is a formation of hydroxy radical (HO), although this process requires the interaction of H_2O_2 and suitably chelated iron, which is unlikely to occur in vitro [1]. Indeed, thermodynamic calculations have been used to claim that HO cannot be formed from peroxynitrite [9]. Therefore, it is most likely that the endothelial apoptosis by SIN-1 is due to a direct cytotoxic action by peroxinitrite. We used SIN-1 as a provider of peroxinitrite. The production of peroxinitrite by SIN-1 was confirmed by the formation of rhodamine from dihydrorhodamine, because rhodamine formation is directly mediated by peroxinitrite [12]. We also studied the effect of NO on the endothelium using SNAP as a source of NO and found that NO per se was not toxic to the cell. If NO were toxic to the cells, SOD might act as a toxic agent as well, because SOD reaction with O_2^- would potentially elevate a steady state concentration of NO. But that does not seem to be the

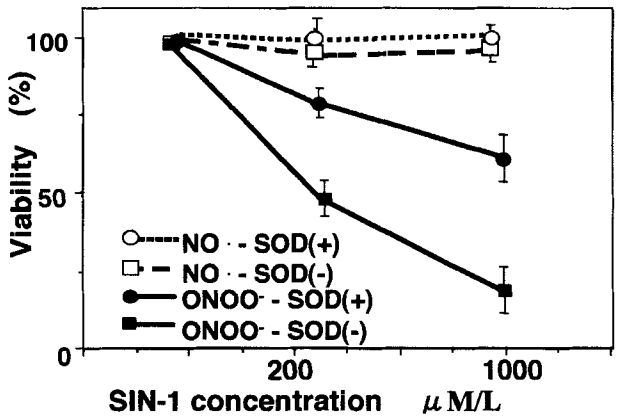

Fig. 5. The cytotoxicity of the cultured porcine aortic endothelial cells. The MTT assay was used in the determination of the cell. The viability of the control cells when they were incubated with SNAP (*open rectangle*) in the concentrations of 200 and 1,000 μM were 97.9 ± 1.5% and 97.7 ± 1.5%, respectively, which did not significantly differ from those of the transfected cells with SNAP (*open circle*, 98.2 ± 1.5% and 98.2 ± 1.4%, respectively). When the control cells were incubated with SIN-1, the cytotoxicity (*closed rectangle*) was 78.5 ± 5.5% and 47.4 ± 9.0%, respectively. On the other hand, the cytotoxicity of the cells transfected with h-Cu, Zn-SOD (*closed circle*) was 88.5 ± 3.5% and 76.0 ± 4.0%, respectively, in the concentrations of 200 and 1,000 μM of SIN-1

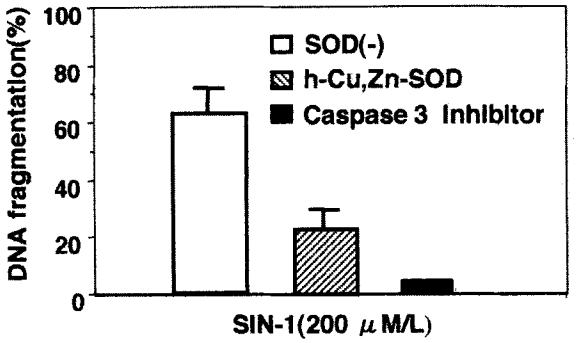

Fig. 6. DNA fragmentation ratio measured by flow cytometry. The cells were incubated with SIN-1 of 200 μM. The cells transfected with h-Cu, Zn-SOD (*hatched*) showed less fragmentation than the control cells (*blank*). The caspase-3 inhibitor significantly suppressed DNA fragmentation (*filled*)

case. Rather, the value of SOD is to prevent the reaction of NO and O_2^- to form $ONOO^-$. This finding is consistent with the reports of others [18].

The apoptosis-inducing effect of peroxinitrite may be mediated by the ability of this compound to initiate various biological responses, including a reaction with DNA by degrading deoxyribose. Other conceivable mechanisms of ROS-inducing endothelial apoptosis may be an association with an alteration in the cytosolic calcium concentration. It is known that oxidative interaction of Ca^{2+}-ATPase of the plasma membrane causes an increase in cytosolic Ca^{2+}, which may subsequently activate proteases, phospholipases and endonucleases [19]. Indeed, there has been evidence that a direct administration of calcium induces apoptosis of the cells [10]. A recent report has documented that increased cytosolic Ca^{2+} induces the binding of calcineurin with Bcl-2, resulting in a loss of its apoptosis-inhibiting capability [24].

In any event, an increase in the scavenging activity of O_2^- is an effective strategic approach to reduce the cytotoxicity. It is therefore important to achieve an effective level of the cytosolic SOD in order to prevent apoptosis. For this purpose, intravenous administration of SOD may not be sufficient, probably because of its short half-life in addition to its insufficient recruitment in the cytoplasm, and it is usually degraded rapidly [11]. Thus, the genetic induction of SOD into the endothelium is an effective way to increase its geological and functional availability for the cells to cope with the burden of ROS. The efficacy of the administration of SOD has been investigated by many researchers with varying results [3, 15]. Such variation in the observations is probably due to the types of cells used or to the difference in the methods of administration of SOD into the cells. However, recent reports have shown that Cu-Zn SOD can be successfully delivered genetically into the hepatocytes in vivo [16, 17].

As is shown in this study, a direct burden on the cells because of large amounts of ROS resulted in necrosis as a characteristic end point of cytotoxicity. The apoptosis was a prominent feature particularly when the cells were incubated with SIN-1 at its intermediate concentration of around 200 μM. Indeed, it has been documented that apoptosis is triggered by oxidants especially when the cells are exposed to low levels of ROS [2, 3, 7]. Theoretically, the apoptotic cells can be effectively cleared by neighboring phagocytes, and the catalytic products could be recycled in the in vivo settings [20]. However, because no such phagocytic cells are present in the in vitro settings, the apoptotic cells would ultimately develop necrosis instead. Therefore, it seems probable that in the mechanism of the endothelial damage caused by ROS, the apoptotic process is an initiating event prior to necrosis unless the ROS is loaded excessively.

Although the signal transduction of the endothelial apoptosis is not well understood, the fact that caspase-3 inhibitor effectively prevented apoptosis indicates that caspase-3 is an important enzyme in the apoptotic process. Among the various types of caspases, caspase-1 and capase-3 are the two best defined, apoptosis-related enzymes. Recent observation has shown that caspase-3 plays an essential role in the apoptotic process, particularly for receptor-mediated apoptosis by Fas-Fas ligand [13]. In our preliminary observation using a monoclonal antibody for mouse Fas antigen, we found that the PAEC failed to show a significant expression of Fas on the cell surface (data not shown). Therefore, it is assumed that at least Fas-mediated apoptosis is not a major factor involved in the apoptotic process caused by O_2^-; rather, ROS may induce endothelial apoptosis either directly or indirectly. Nevertheless, caspase-3 plays an important role in the apoptotic cascade as a common pathway channel for various apoptotic signals. The fact that the caspase-3 inhibitors suppressed apoptosis to a significantly greater extent than SOD transfection indicates the possibility of other undefined pathways of apoptosis that are not protected by oxygen radical scavengers alone.

In conclusion, it is proven that increasing a radical scavenging activity in the cytoplasm successfully prevents apoptosis, which can be achieved by the use of the transfection of h-Cu, Zn-SOD into the endothelium. To the best of our knowledge, this is the first report to demonstrate the effectiveness of genetically transfected SOD in the prevention of O_2^- or peroxynitrite-induced endothelial apoptosis. We believe that such a genetic engineering method is an option to prevent the ischemia-reperfusion injury of the endothelium. We also consider that this novel approach may be applicable in organ transplantation, for example, in xenogeneic organ transplantation.

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