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ORIGINAL ARTICLE

Transplantation of organs is transplantations of donor DNA: fate of DNA disseminated in recipient

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Summary

Microchimerism after allogeneic organ transplantation has been widely documented using DNA identification techniques. However, the question as to whether the detected donor DNA is present in the surviving donor passenger cells, recipient macrophages phagocytizing rejected donor cells, or dendritic cells (DC) internalizing donor apoptotic bodies or cell fragments has not been answered. We provide evidence that allogeneic organ transplantation is followed not only by cellular microchimerism caused by release of graft passenger cells but also dissemination of donor DNA from the ischemic rejecting graft cells and its internalization in recipient DC. The high levels of donor DNA at the time of heart rejection were inversely proportional to the concentration of donor passenger cells detected with use of flow cytometry. Depending on the type of graft, the kinetics of DNA distribution in recipient tissues were different. Immunosuppressive drugs attenuated the rejection reaction and release of DNA from grafts. Allogeneic but not syngeneic donor DNA fragments were found in recipient splenic DC-enriched population. Interestingly, that donor DNA fragments could be detected in recipient tissue at high levels on day 30. This challenges the notion that fragments of DNA are immediately cleaved by cell plasmatic enzymes. The biologic significance of our findings is not clear. We speculate that donor DNA fragments in recipient DC may play a, so far unknown, role in the immunization/tolerance process to allogeneic antigens.

Introduction

Transplanted vascularized organs shed passenger cells, normal constituents of whole organs, which migrate to recipient lymphoid tissues and produce microchimerism. These cells lysed by recipient cytotoxic cells release cellular organelles into the recipient circulation. In addition, warm and cold ischemia as well as immune rejection of the transplanted organ or tissue brings about destructive changes in the graft parenchymatous cells. Fragments of disintegrated cellular organelles are phagocytized by recipient scavenger cells located in lymph nodes, spleen and liver and digested. Some fragments are incorporated into dendritic cells (DC) and processed [1]. Donor DNA is present in the ingested cellular debris. In sex-mismatched male to female transplantation, the Y-chromosome [sex-determining region Y (Sry)] can be detected using specific primers. Donor DNA identified with this assay was found in blood cells after gut [2,3], kidney [4–6], and liver [7] transplantation. A standard amount of DNA is used after extraction from recipient blood cells and tissue biopsies. The question arises as to whether the detected donor Y-Sry fragment is present in the surviving donor cells, the recipient macrophages phagocytizing rejected donor cells or recipient DCs internalizing donor-origin apoptotic bodies or cell fragments [8]. The knowledge of the fate of donor DNA distributed in passenger cells and in fragments of disrupted nuclei as well as the role of recipient cells internalizing donor DNA could give some insight into the mechanism of graft

destruction and immunization or tolerance to donor antigens. Moreover, a futuristic question may be posed as to whether donor DNA fragments could enter the nuclei of recipient DCs and get incorporated into their genome, especially in individuals with viral infections.

In this study, we provide evidence that allogeneic organ transplantation is followed by 'seeding' of donor DNA from the rejecting graft cells and its internalization in recipient macrophages and DCs in lymphoid organs. Immunosuppression with cyclosporin A (CsA) and tacrolimus (FK506) prolonged retention of donor DNA in recipient tissues and DCs.

Material and methods

Animals

The BN ($RT1^n$) male rat tissues were transplanted into LEW ($RT1^l$) female rats. In a syngeneic combination, LEW male to LEW female grafts were performed.

Types of grafts

Three types of grafts were used: bone marrow cells (BMC), hearts, and skin fragments. About 10^7 of BMC were infused intravenously. These cells lodged in lymphoid organs where the rejection process led to their disintegration. Heart grafts were immediately vascularized and perfused. Their rejection was followed by shedding of cellular debris to blood circulation. Skin grafts were vascularized after 3–5 days and only at that time could most of the cellular debris be released to blood circulation. The types of graft and different kinetics of their rejection were reflected by different pattern of donor DNA release.

Experimental setting

The experiments were carried out in eight groups ($n = 3$–5 rats in each group) (Table 1).

Table 1. Experimental setting.

	Graft		Treatment		Follow up
Group	Syngeneic	Allogeneic	CsA	Tacrolimus	(time, days)
1	+	–	–	–	7
2	+	–	–	–	14
3	+	–	–	–	30
4	+	–	+	–	30
5	+	–	–	+	30
6	–	+	–	–	7
7	–	+	+	–	30
8	–	+	–	+	30

CsA, cyclosporin A.

Donor cell identification

To detect BN donor cells present in recipient blood and lymphoid tissues, cells were isolated from LEW recipient blood, spleen, lymph nodes, and bone marrow, stained with OX27 monoclonal antibody specific for BN phenotype and evaluated by flow cytometry. Staining with OX6 [major histocompatibility complex (MHC) class II] and ED1 (macrophages) antibodies allowed to discriminate the dendritic $OX6^+ED1^-$ cells from macrophages and T lymphocytes. For identification of T cells monoclonal antibodies against W3/13, of B cells OX12 and of migrating DCs OX62 were used. All antibodies from Serotec, Oxford, GB.

Harvesting of recipient tissues for DNA

Donor DNA, was extracted from recipient blood, bone marrow, skin, spleen, mesenteric lymph nodes cells, and liver and heart tissue, as well as nonscavenging cells as hepatocytes.

Isolation of splenic dendritic cells

Splenic DC were investigated for the presence of donor DNA. Isolation method recommended by NIH was used [9]. Step 1: Collagenase D (Boehringer, Warsaw, Poland) digested recipient splenocyte suspension was centrifuged 10 min 280 *g*, 4 °C and the cell pellet was resuspended in 5 ml of dense bovine serum albumin (BSA; Sigma, Poznan, Poland) overlaid with 1.5 ml of 4 °C RPMI-1640 (Life Technologies, Warsaw, Poland) medium and centrifuged 15 min 9500 *g*, 4 °C. Step 2: Cells from the interface were collected and resuspended in RPMI-5 (Life Technologies) to 10^7 cells/ml, plated 4 ml per 60-mm dish and incubated 90 min (step 1). The nonadherent cells were removed by gently washing with 37 °C RPMI-1640. Then, pellet was resuspended in 1 ml of RPMI-5 and 50 sheep erythrocytes (WSiS Warsaw, Poland) coated with rabbit antierythrocyte serum (Sigma), were added per leukocyte. Step 3: The cell suspension was gently mixed to break up large aggregates and overlaid on 5 ml dense BSA, centrifuged and interface cells were collected. Such suspension yielded $6–9 \times 10^5$ per spleen after of syngeneic and $2–4 \times 10^6$ after allogeneic transplantation. This DC-enriched population contained more than 60% of $OX6^+$ 30% of ED1, 12% of OX62, and 15% of W3/13 cells.

Analysis of DNA from recipient tissues

DNA was extracted from all cell and tissue specimens for detection of donor Y-Sry fragment. Genomic DNA was prepared according to the protocol of the manufacturer's

instructions (QIAamp DNA Kit; Qiagen, Wroclaw, Poland). Quality of isolated DNA was checked electrophoretically on 1% agarose gels with ethidium bromide. Quantification of DNA was performed in triplicates spectrophotometrically in capillary cuvettes on GeneQuant (Amersham Pharmacia Biotech, Vienna, Austria). Polymerase chain reactions (PCR) always contained 5 ng of DNA, 25 pmol of each rat Y-Sry-specific primers 5′-GAGAGAGGCACAAGTTGGC-3′, 5′-AATACCAGTGG ATGTGATGCGG-3′ and 12 μl of reaction mixture HotStarTaq Master Mix Kit (Qiagen), final volume 25 μl. Amplification was carried out in thermal cycler (DNA Engine; MJ Research, Boston, MA, USA) according to the protocol: 5 min at 94 °C, followed by 35 cycles of 94 °C 30 s, 54 °C 30 s, 72 °C 30 s, finally 72 °C 5 min. Each 1 μl PCR product (about 120 bp) was electrophoresed through ultrathin 12.5% polyacrylamide gel (PAGE; Phast System, Amersham Pharmacia Biotech) and silver stained (Silver Staining Kit; Amersham Pharmacia Biotech). The gels were scanned and analyzed by OneDscan software (Syngen, Cambridge, UK). The differences in optical density (OD) values of triplicates of each sample did not exceed 5%.

Isolation of hepatocytes

Hepatocytes were isolated from recipient livers according to Seglen [10].

Statistical evaluation

Data were expressed as mean values from 3 to 5 experiments. Differences between groups were analyzed using the Mann–Withney *U*-test. The significance of differences was set at $P < 0.05$

Results

Donor phenotype cells in recipient tissues

The donor phenotype (OX27-positive, specific for BN but not LEW) cells were detected 7 days allogeneic after heart transplantation (group 6) in recipient blood cells in 4.3 ± 2.8%, in spleen in 1.9 ± 1.2%, and in bone marrow in 0.3 ± 0.3%. Thirty days after allogeneic heart transplantation and treatment with CsA (group 7) donor type cells were not detected.

Donor DNA in extracts of recipient tissues

The presence of donor Y-Sry fragment was documented in all investigated allogeneic and syngeneic recipient tissues (Figs 1 and 2) however, at different levels. The number of donor DNA copies (expressed in OD units) was close to zero in blood cells, reaching the values of 2–6 in all other specimens.

Groups 1, 2 and 3 (syngeneic grafts, 7, 14 and 30 days): The OD of donor DNA fragments was on day 7 high after heart but low after BMC and skin transplantation (Fig. 1). About 14 and 30 days after heart transplantation the OD values were lower than after 7 days. There was no donor DNA in blood cells but its level was still relatively high after skin grafting in BMC, skin, nodes, and spleen and after BMC transplantation in recipient BMC and spleen. The highest values were found in all groups in the spleen.

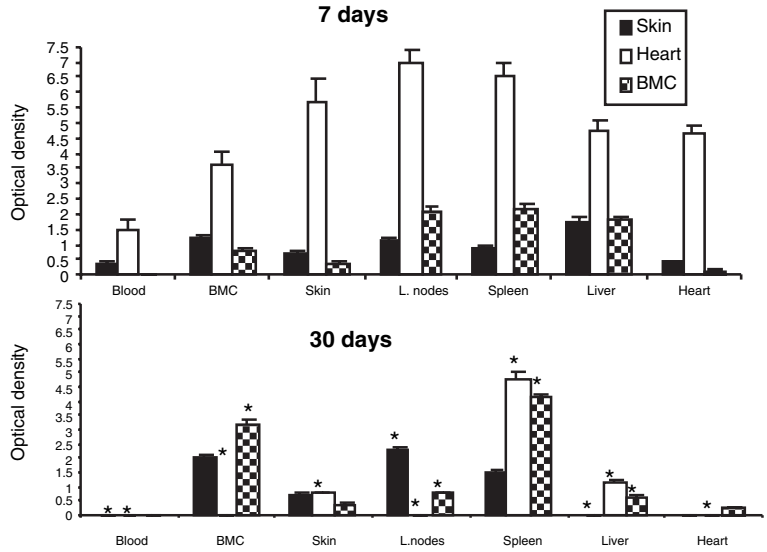

Figure 1 Mean of optical density (OD) values of donor sex-determining region Y (Y-Sry) DNA fragment in recipient tissues 7 and 30 days after syngeneic bone marrow cells (BMC), heart, and skin transplantation (±SD). *Differences between 7 and 30 days, $P < 0.05$.

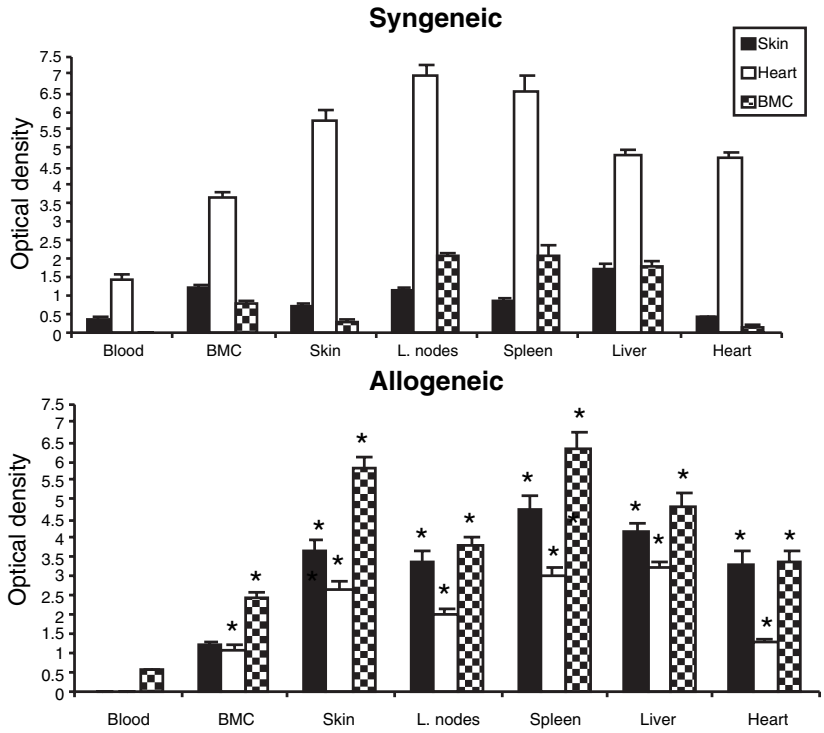

Figure 2 Mean of optical density (OD) values of donor sex-determining region Y (Y-Sry) DNA fragment in recipient tissues 7 days after syngeneic and allogeneic bone marrow cells (BMC), heart, and skin transplantation (±SD). *Differences between allogeneic and syngeneic, $P < 0.05$.

Group 6 (allogeneic grafts, 7 days): The OD values after allogeneic heart transplantation were significantly lower than after syngeneic grafts ($P < 0.05$) but higher after grafting of BMC and skin (<0.05) (Fig. 2).

Groups 4 and 7 (syngeneic and allogeneic grafts, 30 days of CsA): Treatment of allogeneic recipient with CsA resulted in high OD values in all recipient investigated tissues but blood cells and heart (Fig. 3). The highest values were found in the spleen. The values in the allogeneic group were higher than in the syngeneic graft group ($P < 0.05$).

Groups 5 and 8 (syngeneic and allogeneic grafts, 30 days of tacrolimus): Treatment of allogeneic recipient with tacrolimus provided values significantly higher than in the syngeneic group ($P < 0.05$). Again, the highest values were found in the spleen.

Donor DNA in recipient splenic DC-enriched population

The Y-Sry fragment assay revealed the presence on day 7 and 30 of donor DNA in recipient DC after allogeneic but not syngeneic transplantation. Treatment with CsA and tacrolimus for 3 days resulted in retention of donor DNA fragments in both the syn- and allogeneic groups (Fig. 4). The population obtained in isolation step 2 containing lymphocytes forming E-rosettes did not reveal presence of donor DNA.

Donor DNA in nonscavenging cells

No donor DNA was detected in recipient hepatocytes.

Discussion

In our previous studies on microchimerism after heart transplantation we found that while the number of detectable donor cells in recipient blood and lymphoid tissues decreased in time, the number of Sry-fragment copies in the PCR assay increased [11]. The presence of Sry-fragment was low as long as the heart allograft rejection was controlled by CsA. Cessation of immunosuppressive therapy resulted in appearance of donor Y-Sry fragment in all recipient tissues [11]. The present study was extended to syngeneic and allogeneic sex-mismatched transplants. In addition, recipient cell population containing donor DNA was identified.

Donor Sry-PCR product was found, in all investigated recipient tissues. It was not detected in parenchymal cells as hepatocytes but was evidently present in the splenic DC-enriched population.

After syngeneic grafting, donor DNA fragments were detected in recipient tissues at highest levels following heart transplantation. This was most likely caused by washout of the disintegrated, ischemically damaged

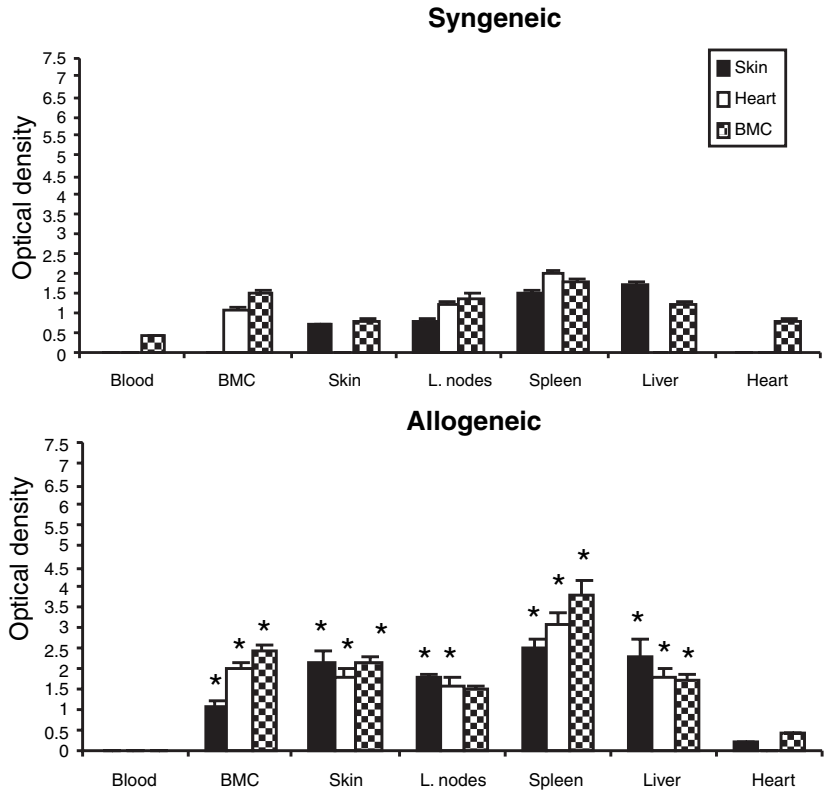

Figure 3 Mean of optical density (OD) values of donor sex-determining region Y (Y-Sry) DNA fragment in recipient tissues 30 days after syngeneic and allogeneic bone marrow cells (BMC), heart, and skin transplantation and treatment with cyclosporin A (±SD). *Differences between allogeneic and syngeneic, $P < 0.05$.

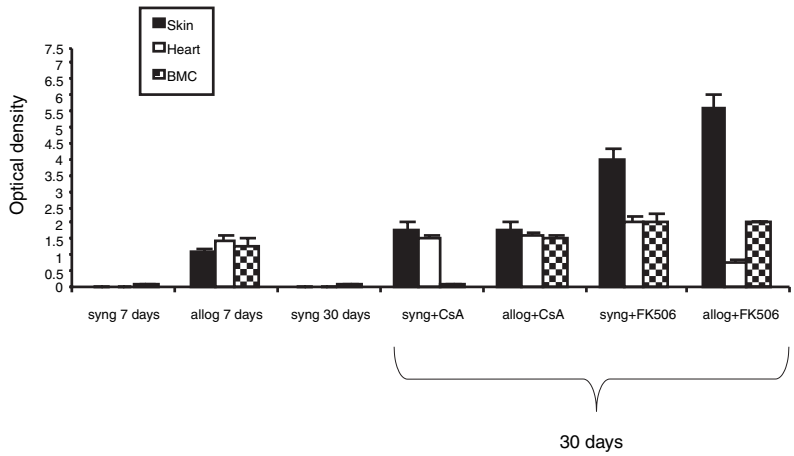

Figure 4 Mean of optical density (OD) values of donor sex-determining region Y (Y-Sry) DNA fragment in recipient splenic dendritic cells (DC)-enriched population 7 and 30 days after syngeneic and allogeneic bone marrow cells (BMC), heart, and skin transplantation and treatment with cyclosporin A or tacrolimus (±SD).

endothelial cells. The DNA levels were lower after skin grafting, what may be explained by late vascularization of the transplant. Transplantation of BMC was followed by their immediate disappearance from blood circulation and lodging to the lymphoid organs and subsequently high DNA level. Interestingly, 30 days after heart

transplantation the DNA values decreased, as there was probably no more shedding of the damaged cell debris. The OD values after BMC transplantation were high as the BMC remained in the lymphoid organs. Skin grafts were still shedding DNA on day 30 from the remodeling tissue.

Seven days after allogeneic heart transplantation the DNA levels were lower than in the syngeneic group. This was probably the result of hypoperfusion of the rejecting graft and subsequently less shedding of cellular debris. As the number of passenger cells was similar in the syngeneic and allogeneic grafts, it can be inferred that the differences in donor DNA level in recipient tissues depended on DNA from the rejecting parenchymatous cells. The BMC transplantation was followed by higher levels of detected DNA in lymphoid tissues compared with syngeneic grafts. This may be accounted for by rejection of allogeneic BMC in lymphoid organs. Rejection of allogeneic skin grafts brought about donor DNA values even higher than after heart transplantation, although the mass of both transplants was similar. The differences could be attributed to different kinetics of rejection.

Treatment of recipients with CsA or tacrolimus resulted in retention of donor DNA in all lymphoid organs at high levels irrespective of the type of graft. The DNA levels were lower than in the acutely rejecting recipients what may be explained by attenuation of rejection reaction by immunosuppressive drugs.

The detected donor DNA fragments could be located in the surviving donor passenger cells or were engulfed by the recipient tissue macrophages, dendritic, or parenchymal cells. It has been reported that macrophages indiscriminately phagocytize damaged cells and their debris, whereas DCs take up DNA, RNA, and oligonucleotides with unclear mechanism [12]. An 80-kDa surface protein that mediates the transport of oligonucleotides was identified [13]. A common binding site for internalization of DNA was found on granulocytes, monocytes, and lymphocytes [14]. In our studies, the DCs contained allogeneic but not syngeneic donor DNA. Donor genetic material present in a fragment of nucleus or chromosome could be internalized in the recipient DCs. This process could be mediated by macropinocytosis, receptor-mediated endocytolysis via C-type lectin receptor, Fcγ-receptor I and II or engulfment involving CD36 and αvβ3 or αvβ5 integrins [15]. However, this mechanism does not discriminate between the allogeneic and own antigens. Thus, the recipient MHC molecules should most likely be the recognizing structures for the donor chromosome fragments. It was observed that mice bone marrow-derived DCs uptake cell fragments into MHC class II-rich compartments [8]. The parenchymal cells, in our studies hepatocytes, did not internalize free DNA. Thus, donor DNA detected in recipient organs was presumably located foremost in their DCs.

Taken together, this study provided evidence for release of donor DNA both from ischemic syngeneic as well as rejecting allogeneic grafts. Depending on the type of graft, the kinetics of DNA distribution in recipient tissues was different. Immunosuppressive drugs attenuated the rejection reaction and release of DNA from grafts. Allogeneic but not syngeneic donor DNA fragments were found in recipient splenic DC-enriched population. Interestingly, donor DNA fragments could be detected in recipient tissue at high levels for periods as long as 30 days. This challenges the notion that fragments of DNA are quickly cleaved by cell plasmatic enzymes. The high levels of donor DNA at the time of heart rejection were inversely proportional to the concentration of donor passenger cells detected with use of flow cytomerty. The biologic significance of our findings is not clear. We speculate that donor DNA fragments in recipient DC may play a role in the immunization/tolerance process to allogeneic antigens. The DNA integration may be seen as an ongoing natural process, which can be harnessed to artificially introduce modification to a cell genetic content [16]. Integration of DNA from apoptotic bodies [17] and repair chromosomal lesion by insertion of mitochondrial DNA have been described [18].

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