Growth potential of aortic autografts and allografts: effects of cryopreservation and immunosuppression in an experimental model

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Abstract

Objective: An animal model has been used to evaluate the potential of growth of vascular autografts and allografts, and the effects of cryopreservation, rejection and immunosuppression on this growth. Methods: In 35 animals (seven groups of five female NZW rabbits; age 5–6 weeks; weight 1.1 kg), a graft interposition was performed at the level of the infrarenal aorta. Different groups included fresh autografts, fresh and cryopreserved consanguineous allografts (donor: litter sister), fresh and cryopreserved immunosuppressed (IS) consanguineous allografts (receiving cyclosporin 10 mg/kg per day) and fresh and cryopreserved allografts. Animals were allowed to grow normally and were sacrificed at the mean weight of 2.89 kg. We studied the growth of the native aorta and of the graft and calculated the growth ratio (growth of the graft/growth of native vessel). Grafts and adjacent aorta were histologically studied. Results: Growth of the graft was normal (mean ratio 1.08; S.D. = 0.21) for autografts, and for fresh and cryopreserved IS consanguineous grafts. Growth was absent (mean ratio 0.12; S.D. = 0.15) for fresh and cryopreserved allografts (P = 0.0001). In consanguineous grafts without IS, growth was absent or normal, presumably according to genetic compatibility, but never intermediate. Histological study showed normal optic microscopic aspects when growth was normal and, when growth was absent, aspects compatible with rejection including mainly intimal hyperplasia and medial thinning. Conclusions: (1) Normal growth of arterial autografts was confirmed; (2) cryopreservation did not prevent potential growth of an arterial graft; and (3) in an allogenic situation, without IS, an aortic graft, fresh or cryopreserved, never showed any growth potential. © 1997 Elsevier Science B.V.

Keywords: Transplantation, homologous; Cardiac surgery; Heart valve replacement; Tissue preservation

1. Introduction

Since the first implantations [28], human allograft valves and vessels have been widely used for surgical treatment of both acquired and congenital cardiovascular diseases mainly because of an apparently better durability than any other biological valves orvalved conduits [2,3,23,24]. Relations between their viability at time of implantation and long-term results are the subject of debate and remain poorly understood. Further studies [10,15,27,36,39,41] have addressed the problem of immunogenicity of the different cellular allograft components: fibroblasts [2,14,24], endothelium [18,19,37–39] and smooth muscle cells [31]. Some authors have suspected allograft rejection in humans [6] and recommend immunosuppression, whereas others argue for implantation of only freshly harvested tissue [7,26]; hence, almost no evidence of rejection has been described [34]. Nevertheless, it is plain that long-term tissue degeneration exists and, therefore, interest in the use of pulmonary autograft as aortic valve replacement is increasing [8,9,29].

Clinically, cryopreserved human valvar allograft conduits implanted in children do not show any growth
2. Materials and methods

Female NZW rabbits from known parentage were used for all the study. Donors, recipients, controls and autografted animals were operated upon between 5 and 6 weeks of age, at a mean weight of 1.1 kg (S.D. = 0.15). The rabbits were either litter sisters (hereafter called consanguineous) or had different parents and grandparents.

2.1. Aortic graft procedure

General anaesthesia with spontaneous ventilation was administered with mixed ketamin (5–7.5 mg/kg) and chlorpromazine (0.5–0.75 mg/kg) administered by intramuscular injection and repeated after 30–60 min if necessary. The procedures were performed in a completely sterile fashion. The infrarenal aorta was approached via a lateral retroperitoneal incision and dissection under an operating microscope. The diameter of the aorta was measured immediately after the procedures were performed with a calibrated eyepiece in the microscope and directly with a scaled-down version of a cantilever transducer calibrated between 0 and 4 mm. All the measurements, at this step or during harvesting or sacrifice were repeated three or four times and averaged. The blood pressure was not recorded invasively but no measurement was done if the vital signs were not strictly normal and stable. The aorta was clamped between two posterior branches (leaving them open to avoid paraplegia [13,25]), usually at the level of the inferior mesenteric artery (which on the contrary could be temporarily clamped). The aorta was then divided. If a fresh autograft was performed, a segment of approximately 2.5 mm of the vessel was explanted, rinsed in 4°C sterile tissue culture medium (RPMI 1640; Life Technologies, Cergy, France) and immediately reimplemented. Two end-to-end anastomoses were performed with interrupted 9/0 nylon sutures and the aorta was unclamped. If any other graft was performed, the aorta was simply divided and the graft interposed and sutured the same way.

2.2. Graft harvesting

The procedures were performed under the same anaesthesia protocol. A median laparotomy was performed and the infrarenal aorta dissected under the operating microscope. The diameter was measured as described above. The infrarenal aorta was then harvested from below the renal arteries to the iliac bifurcation. Two grafts were usually obtained from one donor.

2.3. Treatment of the graft: study groups

The aortic segments harvested from a donor (or from an animal due to receive a fresh autograft) were immediately placed in 4°C RPMI 1640, as in clinical practice for our harvested human allograft valves. They were then immediately reimplemented either in the donor itself for a fresh autograft, or into another animal either from a different parentage for a fresh allograft, or a litter sister for a fresh consanguineous allograft. Alternatively, the tissue was treated and cryopreserved in the same manner as human valve allografts are treated in our tissue bank as follows.

Grafts were immediately transferred to the tissue bank in cold sterile RPMI. They were submitted to 6 h of sterilisation in a medium containing polymixin (100 mg/l), vancomycin (50 mg/l), cefoxitin (300 mg/l) and lincomycin (120 mg/l), this being the same sterilisation time used for human valves sterilily harvested during a cardiac transplantation, for instance. They were subsequently cryopreserved in a cryoprotective medium composed of 10% dimethylsulphoxide (DMSO) and 90% reconstituted 4% human albumin (20% human albumin and Ringer lactate), with a controlled
rate of freezing, and placed in the vapour phase of liquid nitrogen. When required, they were thawed rapidly by immersion in 37°C water and rinsed from DMSO as soon as possible, then transferred back to the microsurgical laboratory in RPMI at 4°C.

They were subsequently reimplanted either in a litter sister of the donor for a cryopreserved consanguineous allograft, or in an animal from different parentage for a cryopreserved allograft. In all the cases, the graft were not submitted to any warm ischaemia. The cold ischaemic time before implantation, or before and after cryopreservation and thawing, never exceeded 4 h.

Of the animals receiving a fresh or cryopreserved consanguineous allograft, half of them received postoperatively an immunosuppressive treatment started the day after surgery and composed of 10 mg/kg per day of cyclosporin (Sandimmum, Sandoz laboratory). The medication, 5 mg/kg twice a day, was given directly in the mouth of the awake animals in the liquid form used in human practice (100 mg/ml). The study groups are summarised in Table 1. The procedures were conducted until all groups consisted of five animals; 10 more animals had either only surgical exposure and measurement of the aorta (4) or aorta measurement, division and immediate suture [6] to serve as controls.

### Table 1
The study groups and their characteristics

<table>
<thead>
<tr>
<th>Study groups</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
<th>Group V</th>
<th>Group VI</th>
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<td>Graft treatment</td>
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<td>Matching rec./donor</td>
<td>Same animal</td>
<td>Litter sister</td>
<td>Litter sister</td>
<td>No matching</td>
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<tr>
<td>Post-op treatment</td>
<td>Cyclosporin</td>
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2.4. Follow-up

The animals were allowed to grow normally, receiving appropriate nutrition and water. The 10 animals of the IS groups received their medication 6 days a week throughout the follow-up period. Cyclosporin blood levels were not studied, this having been done in other studies (personal data, [33], unpublished data from the authors).

2.5. End of the study

The animals were sacrificed when the rapid growth period was terminated, between 1 and 2 months postoperatively. Under general anaesthesia but this time without sterile care, the infrarenal aorta was approached via a transperitoneal approach (free of adhesion). The diameter of the native aorta just above the operated area was noted, the graft carefully dissected out and measured. The animal was then killed, the graft and the adjacent aorta were harvested and placed in formalin. All the specimens were mounted in paraffin and longitudinal sections were stained with haematoxylin and eosin. For each specimen, six sections were prepared and the two best oriented selected for examination. Again with a calibrated eyepiece and away from the suture zones, the intimal and medial thickness were

### Table 2
Ponderal growth during the first post-operative month (in % of the operative weight)

<table>
<thead>
<tr>
<th></th>
<th>Group I (fresh auto.)</th>
<th>Group II (fresh IS con-sang.)</th>
<th>Group III (fresh con-sang.)</th>
<th>Group IV (fresh allo.)</th>
<th>Group V (cryop. IS con-sang.)</th>
<th>Group VI (cryop. con-sang.)</th>
<th>Group VII (cryop. allo.)</th>
<th>Control</th>
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<td>Ponderal growth (%)</td>
<td>75</td>
<td>73</td>
<td>69</td>
<td>59</td>
<td>77</td>
<td>69</td>
<td>62</td>
<td>89</td>
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<td>Standard deviation</td>
<td>22</td>
<td>17</td>
<td>27</td>
<td>10</td>
<td>18</td>
<td>15</td>
<td>22</td>
<td>17</td>
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<tr>
<td>Statistical significance</td>
<td>NS</td>
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All the animals presented about the same weight gain; the smallest ponderal growth was for groups IV and VII (allografts) but this did not reach significance.
measured and the presence or absence of infiltrating mononuclear cells and the presence of fibrosis were noted (inflammatory responses close to the sutures were neglected). The acquisition of all the data at the end of the study, either surgically or histologically, was done in a blind fashion, the observer being unaware of the group from which the animal or the specimen originated.

2.6. Animal care

Animals received humane care in compliance with the ‘Bonne Pratique de Laboratoires’ (according to the ‘Guide for the Care and Use of Laboratory Animals’ published by the US National Institute of Health). The study was approved by the Ethical Research Committee of our university.

2.7. Acquired and calculated data: statistical analysis

The weights of the animals at operation, at 1 month post-operatively and at death were noted, as well as diameters of the native aorta and the graft at operation and at sacrifice. The gain in weight was compared between the groups, considering the difference between weight at surgery and at 1 month post-operatively because all the animals had not reached exactly the same age at sacrifice (because of practical problems with availability of the Surgical Research laboratory). For the same reason, the absolute growth in diameter of the vessel was calculated and statistically treated but the most accurate measurement of the growth of the grafted vessel was the growth ratio dividing the absolute growth of the graft by the absolute growth of the native aorta for each case. This ratio was 1 if the growth of the graft was exactly the same as the growth of the normal vessel of the animal, 0 if the graft displayed no growth. The histological data were acquired and thickness of both the intima and the media were found to be easily statistically treatable. All these data were treated with the help of a personal computer Apple Macintosh® and the software Statview II (v. 1.03, Abacus Concepts, Berkeley, CA). Multiple one-way analysis of variance (ANOVA; Friedman’s test) was used to evaluate differences between groups. Dunett and Fischer’s $t$-tests were used to evaluate differences within each group. The threshold for statistical significance was $P < 0.05$.

3. Results

At the beginning of the study, two animals suffered from immediate post-operative paraplegia and were killed. The sutured aorta was patent but a posterior collateral occluded. We therefore modified the protocol as previously described and this did not recur. No animal died during the follow-up period, and at sacrifice no graft was occluded, thus permitting collection of all the data for each case.

3.1. Statural growth

The gain in weight during the first post-operative month was comparable between all the groups (Table 2). It is interesting that the two groups in which the growth was lesser, also not significantly, were also the allografts groups. Animals continued to grow normally.
after the first month and were killed at the mean weight of 2.89 kg (S.D. = 0.45). This represented a mean gain of 162% compared with the operative weight.

3.2. Diameter increase of the native aorta

At operation the mean diameter of the aorta was 1.97 mm (S.D. = 0.145). The mean diameter of the host aorta at the end of the study was 2.86 mm (S.D. = 0.23). This represented a diameter increase of 45%. There were no statistical differences between the groups for those values, the diameter increase of the native vessels being identical to the control for all the operated animals, even those which received cyclosporin.

3.3. Diameter increase of the graft

At the first operation the diameter of the graft was similar between all the animals (mean, 1.92 mm; S.D. = 0.137) and almost identical to the diameter of the grafted vessel (1.97 mm; S.D. = 0.145). There was no statistical difference between and inside the groups for those values. At the end of the study, when the animals were killed and the measurements made, major differences were readily noted between the animals of different groups. Firstly, and not easy to quantify, dissection of the graft was always more difficult in the allografts groups (groups IV and VII) where the operated area was more inflamed. The dimension of the graft was found to be equal to or greater than its size when implanted, except in one case of a cryopreserved allograft where the graft at the end of the study was smaller (1.7 mm vs. 1.85 mm at first operation).

3.3.1. Fresh autografts (group I)

The increase in diameter of the autografts was strictly comparable with control aortas and with native vessels of the animals (Fig. 1). The fresh autografts grew from a mean diameter of 1.97 mm (S.D. = 0.14) to a mean diameter of 2.75 mm (S.D. = 0.45). When compared with native aorta in terms of the growth ratio, it appears that all the values were around 1, meaning that the graft and the grafted aorta had exactly the same increase in diameter.

3.3.2. Fresh or cryopreserved allografts (groups IV and VII)

The fresh or cryopreserved allografts exhibited almost no growth at all (Fig. 2). Compared with native aorta, the growth ratio was approximately 0, and compared with autografts (in which diameter increases were normal), the difference was highly significant (P = 0.0006). The cryopreserved allografts showed exactly the same results as the fresh ones, and no differences could be found between groups IV and VII.

3.3.3. Consanguineous grafts without IS (groups III and VI)

Fresh and cryopreserved consanguineous allografts yielded interesting results. The growth of the grafts appeared to be either normal, with a ratio around 1, or absent, with a ratio around 0, but never intermediate. In Fig. 3 these ratios are shown in a scattered manner. Here again the cryopreservation did not introduce any apparent change in the results.

3.3.4. Consanguineous grafts with IS (groups II and V)

Fresh and cryopreserved consanguineous allografts in groups II and V, i.e. animals receiving IS, displayed normal signs of growth (Fig. 4). The increase in diameter of the grafts of these two groups was comparable to the autografts, and hence comparable to the growth of the native aorta. The difference of growth between those groups and the allograft group was highly significant. Again there were no differences between fresh and cryopreserved grafts in these groups either.

3.4. Histological study

Histological aspects of the aorta of the controls and of the native aorta of all the animals, even those which received IS, were normal. Three times only, among
Fig. 2. Growth (increase in diameter) of fresh \((n = 5)\) and cryopreserved \((n = 5)\) aortic allografts compared with growth of the native aorta and with autografts. Allografts exhibited no growth \((P = 0.0006)\).

more than 80 specimens examined, a mononuclear infiltrate was observed, twice in the media, once in the hyperplastic intima. The most striking result was the fact that in all the specimens of the groups which exhibited no growth potential, a considerable thickening of the intima was noted as well as a less significant thinning of the media. The quantitative evaluation of those values reached significance. Measurements of the intima and of the media are summarised in Table 3. In each case with a normal diameter increase, the histological aspect of the graft was noted as normal, resembling the aspect of the host aorta and of the controls vessels. The cryopreservation of the graft before implantation did not result in any change in the histological aspect within comparable groups (II and V; IV and VII).

4. Discussion

The aim of this study was to develop, with an experimental model, an approach to the study of autograft and allograft viability which was not based on cellular viability assays which are difficult both technically and in interpretation of the data. Hence, we concentrated on studying size growth and histological aspects after growth. The animal model we used was the rabbit because we reasoned that a rat model, implying a first operation in a young rat weighing less than 0.1 kg, may have introduced a too large technical bias. For the same reason, we chose to implant a valveless vascular segment of a size identical to the grafted vessel, thus avoiding effects similar to an aneurysm, which is possible with some of the models used by others, for example a composite conduit [12,13]. Strictly speaking, therefore, the results are valid for vascular but not valvular growth. However, the same situation occurs with all the models of heterotopic implantation of valved conduits where at least one of the leaflets of the valve is excised or incorporated in the suture to allow regurgitation [17,19,37], or where the valve is not reimplanted at all [12,13,21].

No pure inbred immunologically identical rabbits were available (such as would have been the case with syngenic rats), thus limiting the results in terms of effects of histocompatibility to comparisons between autografting, allografting (as in clinical practice), and between consanguineous grafts with and without IS. Our first goal was to study the effects of cryopreservation and, in fact, we expected to obtain different results with fresh and cryopreserved grafts from comparable groups. For this reason we did not study the effects of IS on allografting between animals issued of different parentage. This will instead be the subject of further studies.

With these considerations the results of this study are interesting in several ways. Firstly, they confirm Murata’s work on puppies [21] concerning normal growth and viability of autografts. This is important to justify the increased use of pulmonary autograft as aortic valve replacement [9,29,30], especially in infants and children, and the use of autologous arterial tissues in cardiac and vascular reconstructive surgery in children.
Secondly, our study confirms previous work on the effects of rejection and IS in vascular grafting. The aspects of intimal hyperplasia we observed were completely compatible with rejection as shown in a rodent model [27,33]. In this model, where the grafts were placed at the level of the infrarenal aorta in already adult animals, mild immunosuppression with 5–10 mg/kg per day of cyclosporin [33] or photodynamic therapy [16] prevented graft rejection. In an adult rodent model, long-term rejection appears to lead sometimes to aneurysm formation, which did not occur in our study in growing rabbits.

Our results are consistent with observations from clinical practice: small aortic homografts implanted in children and infants do not grow [11], just as the aortic allografts and some of the aortic consanguineous grafts failed to do so in this study. The potential of pulmonary conduits to grow or to dilate in the systemic circulation remains debated [8,11]. Some experimental models seem to show dilation [1,13,20] but in an immunologically different environment, whether experimental or clinical, normal growth has never been reported and this is true of our study also. Confirming in a single model some separate points of several studies on immunogenicity of vascular and valvular grafts [10,15,18,19,31,35–41], we observed: (1) that in an allo- genic environment the grafts were rejected and did not grow; (2) that without IS, a normal growth achieving a normal histology was possible only in some presumably histocompatible consanguineous animals; and (3) that IS with cyclosporin permitted normal growth and a normal histological structure after growth in all studied consanguineous animals.

An important part of our results is the fact that cryopreservation in the condition of this study (i.e. the same short sterilisation process we use in clinical practice for steriley harvested cardiac allograft valves and controlled cryopreservation with albumin and DMSO) did not affect growth or histological results. These results are compatible with most experimental studies...
showing that cryopreservation preserves cellular viability and antigenicity [15,37,39,40]. In our work, the grafts were not sized by use of dilators or conical sizers and were submitted to the minimum of manipulation before processing. It has been suspected that surgical manipulation and desiccation during the processing of human valves have caused damage to the endothelium [18,31] and therefore have decreased its antigenicity. This may explain the fact that in clinical practice, besides a few observations mainly concerning infants or young children [6], the effects of rejection have almost never been clearly studied after implantation of cryopreserved allografts [34]. Under the conditions of our study, cryopreservation did not prevent normal growth of the graft in some of the consanguineous animals and in all the animals subjected to IS. The sterilisation process and the cryopreservation, which could decrease the antigenicity of the grafts, neither prevented any of the rejection signs noted histologically or the absence of vascular growth in the allografts. In fact, whether cryopreserved or not, the allografts never displayed normal growth and structure. In the allograft groups, the aspect of the operated area was more inflamed and, although not significant, the mean gain in weight was lower in the allograft groups.

The implication of these results for the clinical use of human cryopreserved allografts are unknown, but ‘fresh’ implantation of allografts [7,26] in which antigenicity of all the components is present may not improve long-term durability. In the same way, enhancing cell viability by decreasing surgical trauma, by shortening the ischaemic time before preservation or by decreasing the toxicity of the antibiotic solution may not lead to better long-term results. It is suggested by this study that careful cryopreservation can preserve viability and growth potential perfectly but that only tissue matching (which would necessitate at least an international collaboration) or use of immunosuppression (the toxicity of which would probably be too high) could allow preservation of allograft viability after implantation.

In conclusion, the animal model here proposed allowed the study of fresh and cryopreserved vascular grafts in different settings. It confirmed that aortic vascular autografts retain intact their growth potential and therefore a probable normal viability. It showed that cryopreservation does not prevent potential growth of an aortic graft with normal histological structure after growth. The study suggests that in an allogenic environment, cryopreserved or not, an aortic graft would never remain viable after implantation.

The study of vascular growth during animal growth may allow a good approach to evaluation of allograft viability and, in further studies, permit the testing of different preservation protocols as well as different compatibility matching and IS treatments.

Acknowledgements

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References


Appendix A. Conference discussion

Dr C. Yankah (Berlin, Germany): I congratulate Dr Kreitmann and his co-workers for this elegant, excellent, well-designed study. This study addressed two important issues of clinical interest, namely, the growth potential of vascular autografts and antigenicity of fresh and cryopreserved allografts, as well as the effect of immunosuppressive therapy to maintain the viability of fresh and cryopreserved allografts. There is evidence which shows that even with a short-term course of immunosuppressive therapy, one can achieve tolerance of allografts in inbred rat models. The question I would like to address to Dr Kreitmann is, in your control study where you have the allografts treated with antibiotic solution and those with cryopreserved solution, what is the viability of these two groups? Were they viable or non-viable pre- and postoperatively? And how did you define the growth of your cryopreserved vascular autografts and allografts? Was there any histological difference between growth and dilatation in the allografts under immunosuppression as compared to those who were not under immunosuppression. Do you think that immunosuppressive therapy may be necessary and play a role in the use of cryopreserved viable allografts? I thank the Association for giving me the privilege to discuss this paper.

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is, if you did any experiments with younger animals and if there was any difference with older animals?

Dr B. Kreitmann: In this study, the first operation (the graft) was done around the age of 5–6 weeks, and we thought that we would not be able to practice this kind of operation on younger animals because the animals would be below 1 kg and then we would probably introduce a technical bias. So I cannot really answer this point, I am sorry about that. But in fact in the clinical setting there is some evidence that rejection may be stronger in groups of young patients.