Brief Rapid Communication

Effects of Calcium Channel Blockers on Calcium Uptake in Rat Aortic Valve Allografts

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Background. The life span of human aortic valve allografts is finite, and many fail because of cusp rupture or calcification. Subcellular changes occurring in aortic valves in response to transplantation include the uptake of calcium. This study uses a heterotopic rat aortic valve transplant model to determine whether the calcium channel blockers diltiazem and verapamil might attenuate leaflet calcification.

Methods and Results. The 60 rats studied were divided into the following groups: 1) control: valves from normal, unoperated F₁ generation of Lewis and Brown Norway cross (LBNF₁) rats; 2) control: valves from syngeneic transplant combinations (Lewis/Lewis); 3) control: valves from allogeneic transplant combinations (LBNF₁/Lewis, donor/recipient); 4) experimental: valves from allogeneic strain combinations treated with 30 mg/kg per day diltiazem; 5) experimental: valves from allogeneic strain combinations treated with 30 mg/kg per day verapamil. Drugs or saline (controls) were administered with osmotic pumps placed subcutaneously 2 days before transplantation. Animals were killed 3 weeks later, and the valves were harvested and prepared for calcium analysis. Energy-dispersive x-ray microanalysis was used to measure the calcium in a section of one leaflet from each valve studied. Paired t tests showed that allograft valves treated with diltiazem or verapamil contained significantly less calcium than allograft controls treated with saline (p<0.001). When all five groups were subjected to one-way ANOVA, the valves in the allograft control group contained significantly more calcium than all other groups. All other groups were not different from each other.

Conclusions. The calcium channel blockers verapamil and diltiazem were effective in preventing early calcification that occurs in aortic valves after transplantation. Thus, these agents might play a role in prolonging the life of human aortic valve allografts. (Circulation 1992;86:1973–1976)

Key Words • allograft valves • calcification • verapamil • diltiazem • x-ray microanalysis

Human aortic valve allografts undergo structural deterioration after surgery so that by 10 years after implantation, only about one half are functioning normally. This may be due to technical imperfections, but in most cases it is due to primary tissue failure including tissue attenuation and rupture, cusp shrinkage, and/or intrinsic calcification. The degree of calcification of allograft valve leaflets has been shown to be related to the degree of histoincompatibility between strains of rats. Because human donor valves are not tissue-matched with recipients, calcium uptake of the leaflets caused by chronic rejection may be an unrecognized factor influencing valve survival.

The present study was designed to determine whether the process of valve leaflet calcification might be affected by the administration of calcium channel blockers in a model previously described from our laboratory involving aortic valve allografts in inbred strains of rats. Our interest in calcium channel blockers derives from previous reports of their effectiveness in inhibiting the development of atherosclerotic lesions in other animal models of human disease. The model consists of abdominal aortic valve placement in rats with which we and others have extensive experience. It is attractive because of its technical simplicity and because the availability of inbred rat strains allows the genetics to be precisely controlled. Energy-dispersive x-ray microanalysis was used to quantitate tissue calcium content, as we and others have previously reported.

Methods

Animals

Virus-free F₁ generation Lewis and Brown Norway cross (LBNF₁) male rats (weight, 225–250 g) were purchased from Harlan Co. Animals were housed in the virus-free facility except when surgery was being performed. Valves from 60 animals were studied; they were divided into groups as follows: 1) control: normal, unoperated LBNF₁ rats (n=12); treatment: none; 2) control: syngeneic strain combination of Lewis/Lewis rats (n=12); treatment: saline; 3) control: allogeneic strain combination of LBNF₁/Lewis (donor/recipient) rats (n=12); treatment: saline; 4) experimental: allogeneic strain combination rats (LBNF₁/Lewis) (n=12); treatment: 30 mg/kg per day diltiazem; 5) experimental: allogeneic strain combination rats (LBNF₁/Lewis) (n=12); treatment: 30 mg/kg per day verapamil.

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Recipient animals were implanted subcutaneously with osmotic minipumps (Alzet model 2ML4, Alza Co.) filled with drug solution (50 mg/ml) or saline. Transplant surgery was performed 48 hours later. After 3 weeks of treatment, animals were anesthetized, and the transplanted valves were removed and prepared for x-ray analysis. Blood samples were obtained at 1, 2, and 3 weeks in several animals and assayed for verapamil (Med Tox, Inc., St. Paul, Minn.) or diltiazem (National Medical Services, Willingham, Pa.) to confirm that therapeutic levels were maintained (45 mg/ml for diltiazem and 193–333 mg/ml for verapamil in these animals).

The operations and treatments were performed over a period of 6 months. Rats in each shipment were assigned randomly to the treatment groups.

**Surgery**

**Pump implantation.** Animals were anesthetized with metapthane (methoxyflurane) inhalant anesthesia. Osmotic pumps filled with the appropriate solution were placed into a subcutaneous pouch through a small incision over the animal’s scapula. The incision was sutured with 3-0 silk, and the animal immediately recovered.

**Valve transplantation.** Animals were anesthetized with 3.6% chloral hydrate (1.0 ml/100 g body wt i.p.). The heart and ascending aorta were removed from the donor and placed in ice-cold saline. The aortic valve and a short segment of the aorta were dissected from the heart and stored in ice-cold saline while the recipient was being prepared. The abdominal cavity of the anesthetized recipient animal was opened, and the descending aorta was exposed. The aorta, just distal to the renal vessels, was clamped and divided. The donor valve with aorta was anastomosed end to end to the abdominal aorta using 8-0 prolene suture. One cusp was rendered incompetent to ensure complete washout of the sinuses of Valsalva. The clamps were released, the transplant was examined for patency, and the animal was closed in two layers.

**Valve harvest.** Three weeks after the transplant, each rat was anesthetized with 3.6% chloral hydrate (1.0 ml/100 g body wt i.p.) and killed by exsanguination. The valve allografts were rapidly excised for study.

**Preparation of Tissues for X-ray Microanalysis**

Valves were dissected free of connective tissue and immediately placed in a drop of OCT embedding medium (Tissue Tech) on a slice of cork and were then frozen in liquid nitrogen. Tissues were stored in liquid nitrogen until cryosectioned. Frozen sections (8 μm) containing valve leaflet were collected on carbon stubs and placed in liquid nitrogen, then freeze-dried for 12 hours at −40°C, then dried for an additional 40 hours at room temperature. Tissues were lightly carbon coated in an Emtech K-950 to reduce charge.

**Energy Dispersive X-ray Microanalysis**

Analysis was conducted using a Hitachi S2700 scanning electron microscope equipped with a Kevex Delta 1 energy-dispersive x-ray detector. Sample areas of the valve leaflet were selected with a reduced screen scan and analyzed using an acquired time of 100 seconds at 15 kV with beam current set at 7.0 nA and magnification ×500. Five samples from each sample leaflet were counted (Figure 1). All samples were coded so that their identities were unknown to the operator; data were collected blindly.

**Statistical Analysis**

Quantitative data obtained from x-ray analysis (five samples from each of 12 animals in five treatment groups) were subjected to one-way ANOVA using the Duncan multiple range test. With the level of significance set at 0.01, the test has a power of 0.97.

**Results**

The intensity of the calcium peak expressed in counts/100 sec was recorded for each sample. The mean and standard deviation were calculated for each treatment group (Figure 2). The five treatment groups were compared using one-way ANOVA. Valve leaflets from the allogeneic strain combination treated with saline contained significantly more calcium than did those from all other treatment groups. All other groups were not different from one another. The two-group t test showed that the valves from animals treated with diltiazem and verapamil were significantly less calcified than those treated with saline (p<0.001) and that they were not different from normal valves. Valves from syngeneic grafts contained more calcium than normal valves; however, when all groups were considered, they were not significantly different from normal or drug-treated animals.

**Discussion**

These data suggest that diltiazem and verapamil are effective in inhibiting posttransplantation calcium uptake in aortic valves as measured 3 weeks after surgery. Transplantation in syngeneic strain combinations did not cause significant calcification of the valve leaflets; therefore, we presume that calcification in the saline-treated allografts was not due to surgical pressure gradients, or the heterotopic location of the graft but rather to immunologic events.

Both class I and class II histocompatibility antigens have been demonstrated on viable endothelial cells in cryopreserved aortic valves.8 The antigenicity of fresh, cryopreserved, and cold stored rat aortic valves has been confirmed by Lupinetti and coworkers in our laboratory.4,5,9 Others have demonstrated a relation between calcification and the histoincompatibility of donor grafts with recipient animals. For example, Gonzalez-Lavin and coworkers10 demonstrated a greater calcium content in allograft valve leaflets and conduits in transplants between unrelated dogs than between litter mates. Similarly, Lupinetti and coworkers5 showed in a heterotopic rat model that calcification progresses over a period of 3 weeks, then reaches a plateau. The degree of calcification was directly correlated with the degree of histoincompatibility between donor and recipient, suggesting that rejection may play a significant role in calcium uptake. That calcium uptake might be one of the early events in deterioration of heart valve allografts should not be surprising. Dystrophic accumulation of calcium phosphate in injured tissue occurs frequently in nature, especially at sites of dynamic mechanical deformation such as valve leaflets.3,11 The earliest deposits are localized to connective...
**FIGURE 1.** Rat aortic valve as it would appear just before transplantation is shown using scanning electron microscopy (panel A). Arrows indicate edges of leaflets. Bar, 200 μm. Panel B: Sagittal or frontal frozen sections through transplanted aorta and valve collected for x-ray microanalysis. A section adjacent to the section to be scanned was stained with toluidine blue for reference (shown to illustrate how the five samples from each leaflet were selected). Reduced screen is drawn to scale around sample 3. Cartilaginous aortic ring (C) is a useful landmark in locating the valve leaflet on the freeze-dried section (from an animal treated with verapamil). Bar, 100 μm.
of calcium into the arterial wall, which may play a role in the structural deterioration of these tissues. The valves used in this study were fresh, not cryopreserved as are some valves in clinical use, and it is unknown whether this preservation technique would alter the effects of calcium channel blockers.

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