Angioresistance of Thermally Modified Cartilage Grafts in the Chick Chorioallantoic Membrane Model

Background: The chick chorioallantoic membrane (CAM) model allows direct observation of vascularization acutely in explanted or cultured tissues in an immunologically isolated environment. In vivo, angioinvasion of the tissue matrix does not occur in viable cartilage tissue, whereas denatured or nonviable grafts are readily vascularized and/or resorbed.

Objective: To determine, using the CAM model, whether angioinvasion of thermally altered cartilage explants occurs acutely.

Materials and Methods: Porcine septal cartilage specimens were removed from freshly killed animals and divided into 3 groups (n=10): an untreated control group, a group in which cartilage was boiled in isotonic sodium chloride solution (normal saline) for 1 hour, and a laser-irradiated group (Nd:YAG, λ=1.32 µm, 30.8 W/cm², irradiation time=10 seconds). Tissue specimens were then washed in antibiotic solutions, cut into small cubes (approximately 1.5 mm³), placed on the surface of 30 CAMs (7 days after fertilization), and allowed to incubate for an additional 7 days. After incubation, the membranes and specimens were fixed in situ with formaldehyde and then photographed using a dissection microscope.

Results: Examination with a dissecting microscope showed no obvious vascular invasion of the cartilage or loss of gross tissue integrity in any of the 3 experimental groups, although all specimens were completely enveloped by the CAM vascular network. No vascular invasion of the tissue matrix was observed histologically.

Conclusion: These experiments demonstrate that cartilage specimens remain acutely resistant to angioinvasion or metabolism by the immunologically immature CAM whether native unmodified tissue, completely denatured (boiled), or thermally modified following laser irradiation.

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Surgical reconstruction in the head and neck for the correction of congenital, traumatic, or oncologic defects often requires the use of autogenous nonvascularized cartilage grafts harvested from heterotopic sites. Recent advances in biomedical laser technology have led to the development of nonablative surgical procedures that can be used to reshape cartilage without the need for morselization, suturing, or carving. Photothermal heating during laser irradiation with nonablative power densities results in a temperature-dependent acceleration of mechanical stress relaxation within the tissue matrix that allows the cartilage to be reshaped into new stable configurations. Although this technique has prompted a great deal of interest and research focused on determining the mechanism of reshaping tissue viability following laser irradiation has received only limited study.

Although animal models provide the best method to examine the integrated vascular and immunologic responses to implanted materials, the preliminary nature of laser-assisted cartilage reshaping makes such studies at this point impractical. As an alternative to live animals, we used the chick chorioallantoic membrane (CAM) model to examine the effect of laser irradiation and intense thermal heating (boiling) on acute graft viability. The CAM model is a low-cost hybrid ex vivo and in vivo system that allows direct observation of vascularization in explanted or cultured tissues placed on the surface. Furthermore, because the chick immune system is not competent during the first 17 days after
MATERIALS AND METHODS

Fresh porcine septal cartilage was obtained from a local abattoir (Clougherty Packing Company, Vernon, Calif) and harvested as previously described. The specimens were cut into disks 6 mm in diameter and 1 mm thick and divided into 3 groups (n = 10). Negative control specimens did not undergo any thermal modification. Positive control specimens were boiled in saline solution for 60 minutes at 100°C. Laser-irradiated specimens were exposed to Nd:YAG laser (λ = 1.32 mm, 30.8 W/cm²) radiation for 10 seconds. Following laser irradiation, the specimens were rinsed for 45 minutes in antibiotic solutions (amphotericin, 20 mg/L, and gentamycin, 200 mg/L, in phosphate-buffered saline solution) 3 consecutive times.

The specimens from each group were then cut into small cubes (1.5 mm³) under sterile conditions. The CAMs were prepared as previously described, as illustrated in Figure 1A through C. On the fourth day of incubation in a 38°C, 66% humidified incubator (Profi I; Lyon Electric, Chula Vista, Calif), an 18-gauge needle and syringe were used to aspirate approximately 4 mL of albumin and create an air pocket (Figure 1A). On the seventh day of embryo development, a 20-mm-diameter hole was made by cutting and removing the apex of the eggshell (Figure 1B). A Teflon ring was placed on the CAM surface to stabilize and limit the movement of transplanted samples (Figure 1C). While viewing with a dissection microscope (×15), the specimens were gently placed in the center of the retaining ring. A total of 30 cartilage specimens were placed on 30 CAMs. A sterile Petri dish was positioned to cover the hole in the eggshell. The eggs were replaced in a static incubator and allowed to continue development until after fertilization day 14. At the end of this period, the CAMs were removed from the incubator. The specimens were fixed in situ by adding drops of formaldehyde to the central portion of each ring over the cartilage specimen. Using microdissection techniques, the retaining ring was gently dissected free from the membrane and the specimen immersed in a 10% formaldehyde solution. Preserved CAMs were photographed under bright-field microscope (×30 magnification) with a dissection microscope. Following fixation, specimens were serially dehydrated using graded ethanol solutions and subsequently embedded in paraffin. The microsections were sectioned (6 µm thickness) and stained with hematoxylin-eosin and examined microscopically (×10-×40 magnification).

RESULTS

Figure 2A through C is a photographic montage of native, laser-irradiated, and boiled specimens as visualized with a dissecting microscope at ×40 magnification, respectively. Specimens, although completely enveloped by the CAM vascular network, show no gross loss of structural integrity. Figure 3A through C is a photographic montage of stained histological sections of a native cartilage specimen at ×10, ×20, and ×40 magnifications, respectively. No evidence of angiogenesis is observed within the cartilage matrix, although blood vessels are clearly visible in the enveloping CAM. Similar findings were noted in the laser-irradiated specimens and boiled cartilage specimens, where histological integrity of the tissue is maintained.

COMMENT

Xenographic studies can be performed in the CAM because its immune system does not develop until approximately day 17 after fertilization; graft-vs-host reactions do not occur. As a consequence, the CAM can be used to assess the angiogenic properties of tissues following biochemical and physical modifications. Since the membrane and developing blood vessels are directly visible, the CAM permits observation of the developing vascular network. As illustrated in Figures 2 and 3, all specimens retained histological integrity, despite having been enveloped by the CAM vasculature. Gross and microscopic tissue integrity was maintained during the

fertilization, angiogenesis can be studied in the absence of both cellular and humoral immune responses. In the 1970s, the CAM model was used to study angiogenesis in various tissues. Cartilage was observed to resist angiogenesis from the CAM. Although the explanted tissue would be enveloped by the CAM, no vascular invasion of the cartilage matrix was observed histologically. These and other experiments led to the discovery of angiostatic proteins native to cartilage tissues.
7-day incubation period even in specimens boiled for 1 hour. These findings suggest that even nonviable cartilage can acutely resist vascular invasion. Albeit, in the true animal model, thermally modified cartilage grafts would be observed for substantially longer periods in an immunocompetent host. Resorption of denatured nonviable grafts would likely occur along with an intense inflammatory response. Inasmuch as few clinical procedures that involve the heating of cartilage tissue exist, few studies that focus on the viability of thermally modified cartilage tissue have been reported. Although laser-mediated cartilage reshaping uses nonablative power densities, significant tissue temperature elevations of up to 70°C occur. Although clinical trials using laser radiation to reshape cartilage are under way, the safety of this procedure has not been fully established. The focus of this pilot study was to determine whether heated (with laser or via boiling) cartilage grafts would survive in vivo. Although the results of this pilot investigation show that gross and histological structural integrity were maintained without angiogenesis of the tissue matrix, further animal studies will be needed to determine whether tissue viability is maintained. Even though preliminary biochemical studies demonstrate that laser reshaping can be performed with preservation of a significant fraction of chondrocytes within the cartilage matrix, viability in vivo depends on how the host immune system responds to thermally altered tissue regardless of whether it is autologous or heterogeneous. Heat denatures proteins, and these macromolecules may serve as potent antigens, provoking profound host inflammatory response. Vascularization of such tissues would result in resorption.

Although the study of thermal effects in mesenchymal tissues is still in its infancy, tissue modification and engineering of cartilage tissue and cartilaginous frameworks have been extensively studied for more than 2 decades. With growing interest in tissue engineering cartilage autografts, the need for animal model studies is pressing.

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Editor