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Angiogenic Response Induced by Acellular Aortic Matrix In Vivo

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ABSTRACT

In this study, we investigated the angiogenic response induced by acellular aortic matrices implanted in vivo onto the chick embryo chorioallantoic membrane (CAM), a useful model for such investigation. Results showed that acellular matrices were able to induce a strong angiogenic response comparable to that of fibroblast growth factor 2 (FGF-2), a well-known angiogenic cytokine. The angiogenic response was further increased when exogenous FGF-2 or transforming growth factor beta 1 (TGF-β1) were added to the matrices and inhibited by the addition of an anti-FGF-2 or anti-TGF-β1 antibodies. The response may be considered dependent on a direct angiogenic effect exerted by the matrices and in part also by the presence of FGF-2 and TGF-β1 in the acellular matrices.

Key words: acellular matrices; angiogenesis; aorta; chorioallantoic membrane; fibroblast growth factor 2; transforming growth factor beta

Repair and regeneration of injured tissues and organs depend on reestablishment of the blood flow needed for cellular infiltration and metabolic support. Among various materials used in tissue reconstruction, acellular matrices have been recently utilized (Hodde, 2002). Acellular matrices are the noncellular part of a tissue and consist of proteins such as collagen and carbohydrate structures secreted by the resident cells. They can be transplanted without rejection and provide a conductive environment for normal cellular growth, differentiation, and angiogenesis and a framework for tissue regeneration because they are completely replaced by the host tissue (Hodde, 2002). The integration of these matrices into the host tissue may benefit from a microvascular network capable of anastomosing with the host microvasculature, thereby ensuring an adequate supply of blood and nutrients to the implants. In the last few years, acellular matrices have been successfully used to substitute and repair skin, bladder, urethra, small bowel, and skeletal muscle defects (Sutherland et al., 1996; Takami et al., 1996; Parnigotto et al., 2000a, b; Marzaro et al., 2002). Iurlaro et al. (2002) described a new method for the neovascularization of polyurethane meshes from explants of rat aorta. Aortic rings embedded in collagen-permeated polyurethane meshes and cultured in medium supplemented with fetal bovine serum and an angiogenic cytokine, i.e., vascular endothelial growth factor (VEGF), generated microvascular outgrowths that efficiently vascularized the available spaces between polyurethane meshes.

The decellularization procedure of aorta resulted in a complete loss of all cellular structures with minimal damage to the extracellular matrix. Several authors have developed decellularization techniques followed by in vitro reseeding with autologous cells (Cebotari et al., 2000;

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Steinhoff et al., 2000) or allowing ingrowth of host cells into the graft after transplantation (Wilson et al., 1995; Goldstein et al., 2000; Steinhoff et al., 2000; Dohmen et al., 2002). In the case of arteries, the acellular matrix is composed of insoluble collagen, elastin, and glycosaminoglycans from the original vessel. The structural proteins are well conserved and normally arranged (Wilson et al., 1995).

In this study, we investigated the angiogenic response induced by acellular aortic matrices implanted in vivo onto the chick embryo chorioallantoic membrane (CAM), a useful model for studying angiogenesis and antiangiogenesis (Ribatti et al., 2001) and the effects of other acellular scaffolds, such as those obtained form brain (Ribatti et al., 2003).

MATERIALS AND METHODS

Preparation of Acellular Matrix

Thoracic aorta obtained from pig (aged 2 months) was rinsed four times for 15 min in phosphate-buffered solution (PBS) containing 1% antibiotic and antimycotic solution (Sigma Chemical, St. Louis, MO) and treated according to Meezan et al. (1975) to obtain an acellular matrix. Briefly, samples were frozen and thawed four times and were then processed three times as follows: distilled water for 72 hr at 4°C, 4% sodium deoxycholate (Sigma Chemical) for 4 hr, and 2,000 kU Dnase I (Sigma Chemical) in 1 M NaCl for 2 hr. The absence of cellular elements was confirmed histologically (hematoxylin-eosin staining; Merck, Darmstadt, Germany), and acellular matrices were stored in PBS at 4°C until use.

Immunohistochemistry

Five μm thick sections of thoracic aorta and acellular matrix were incubated at 37°C with primary polyclonal antibodies anti-TGF-β1 (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-FGF-2 (Chemicon International, Temecula, CA) diluted 1:200 in 1% BSA for 60 min. Immunostaining was performed by using the Large Volume Dako LSAB (labeled streptavidin-biotin) + Kit/HPR (horseradish peroxidase; Dako, Carpinteria, CA). A preimmune rabbit serum (Dako) replacing the primary antibody served as negative control.

CAM Assay

One hundred fertilized white Leghorn chicken eggs were incubated at 37°C at constant humidity. On day 3 of incubation, a square window was opened in the egg shell after removal of 2–3 ml of albumen so as to detach the developing CAM from the shell. The window was sealed with a glass and the eggs were returned to the incubator. At day 8, eggs were treated with 1 mm^3 acellular aortic matrices implanted on the top of growing CAMs under sterile conditions. In some experiments, matrices were mixed before implantation with 500 ng/embryo of recombinant FGF-2 (R&D Systems, Abindgdon, U.K.), 500 ng/embryo of anti-FGF-2 antibody (Santa Cruz Biotechnology), 500 ng/embryo of recombinant TGF-β1 (Santa Cruz Biotechnology), 500 ng/embryo of anti-TGF-β1 antibody (Santa Cruz Biotechnology), and a mixture of the two antibodies at the same concentrations. Moreover, 1 mm^3 sterilized gelatin sponges (Gelfoam, Upjohn, Kalamazoo, MI) adsorbed with 500 ng/embryo of FGF-2 dissolved in 2 μl PBS or with PBS alone, used as positive and negative control, respectively, were implanted at day 8 on the top of some CAMs, as previously reported (Ribatti et al., 1997).

CAMs were examined daily until day 12 and photographed in ovo with a stereomicroscope equipped with a Camera System MC 63 (Zeiss, Oberkochen, Germany). Blood vessels entering the implants or the sponges within the focal plane of the CAM were counted by two observers in a double-blind fashion at 50× magnification (Brooks et al., 1994). At day 12, CAMs were processed for light microscopy. Eight μm serial sections were cut in a plane perpendicular to the surface of the CAM, stained with a 0.5% aqueous solution of toluidine blue (Merck, Darmstadt, Germany), and observed under a Leitz-Dialux 20 light photomicroscope (Leitz, Wetzlar, Germany).

The angiogenic response was assessed histologically by a planimentric method of point counting (Ribatti et al., 1997). Briefly, every third section within 30 serial slides from each specimen was analyzed under a 144 point mesh inserted in the eyepiece of the photomicroscope. The total number of intersection points occupied by transversally cut vessels (3–10 μm diameter) was counted at 250× at the boundary between the implants and the surrounding CAM mesenchyme in six randomly chosen microscopic fields for each section. Mean values ± standard deviation (SD) were determined for each analysis. Vascular density was indicated by the final mean number of the occupied intersection points. Statistical analysis was performed using Student's *t*-test for unpaired data.

RESULTS

Histological examination of the acellular matrices revealed the absence of cells and a less compact structure (Fig. 1c and d) compared to untreated tissues (Fig. 1a and b). The detergent-enzymatic treatment preserved the presence of FGF-2 and TGF-β1 from tissues as demonstrated by a diffuse strong immunoreactivity in both whole (Fig. 1a and b) and acellular matrices (Fig. 1c and d). No staining was detectable when a preimmune rabbit serum replacing the primary antibodies was used as a negative control (Fig. 1e and f).

Macroscopic observation of CAMs treated with acellular matrices showed that the implants were surrounded by allantoic vessels that developed radially toward the implant in a spoke-wheel pattern (Fig. 2a). The angiogenic response was comparable to that exerted by FGF-2 (Fig. 2b). No vascular reaction was detectable around the sponge treated with vehicle alone (not shown).

Microscopically, acellular aortic matrices were adherent to the chorion without invading the mesenchyme (Fig. 2c). Numerous newly formed blood vessels radially arranged under the implants (Fig. 2c and d) and, in some points, invaded the chorion and merged inside the aortic matrices over the epithelium (Fig. 2e).

The macroscopic and microscopic observations were confirmed by vessel counts and morphometric evaluations, as shown in Figures 3 and 4. A higher microvessel density was detectable within the CAMs implanted with aortic matrices than in those treated with vehicle alone, comparable to the microvessel density of the CAMs treated with FGF-2. To assess whether the angiogenic response was due to an increased mobilization of endogenous FGF-2 or TGF-β, matrices were added to the CAM in the presence of anti-FGF-2 or anti-TGF-β antibodies. Anti-FGF-2 antibodies reduced the angiogenic response by 60% while anti-TGF-β antibodies by 30%. When the two antibodies were

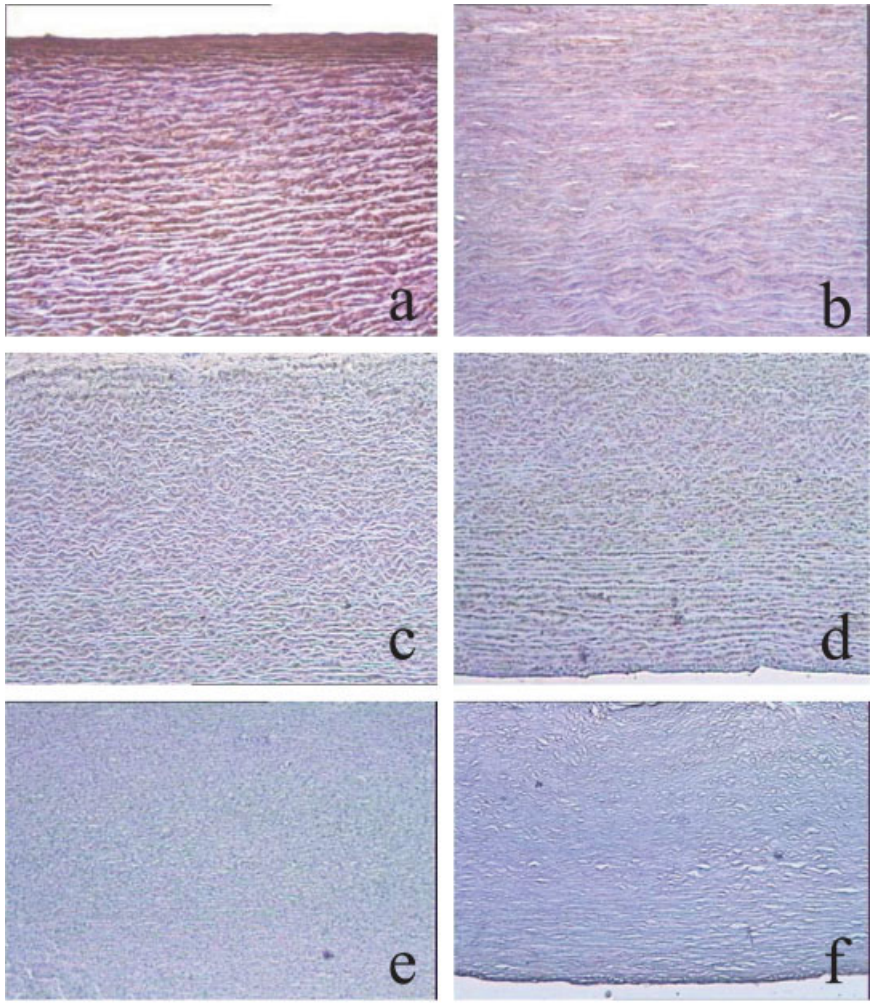

Fig. 1. Sections of thoracic rat aortas (**a** and **b**) and of corresponding acellular matrices (**c** and **d**) immunoreactive to FGF-2 (a and c) and TGF-β 1 (b, d). In **e** and **f**, two negative controls, a preimmune rabbit serum replaces the primary antibodies. Original magnification: 100×.

added together, they acted in a synergistic way, reducing the angiogenic response by 90%. Otherwise, when the acellular matrices were added to the CAM in the presence of FGF-2 or TGF-β, the angiogenic response increased by 30%.

DISCUSSION

In this study, for the first time, we demonstrate that acellular aortic matrices are able to induce a strong angiogenic response, comparable to that of FGF-2 when implanted onto the chick CAM. The newly formed blood vessels grow radially around the matrices and invade them in some points and the angiogenic response may be considered dependent by a direct angiogenic activity exerted by the matrices.

The advantage of using acellular matrices compared to exogenously added growth factors, such as FGF-2, may be attributable to a direct angiogenic activity exerted by the matrices. It is conceivable that extraction techniques may not remove all the factors that impact on angiogenesis. In fact, as demonstrated by immunohistochemical staining, the angiogenic activity may also be dependent on the presence of FGF-2 and TGF-β, as already demonstrated in other experimental conditions (Taipale et al., 1996; Ribatti et al., 2003). Moreover, the main components of the extracellular matrix, such as the fibrous component, consisting of collagen and elastic fibers and the interfibrillary matrix, which contains mainly proteoglycans, solute, and water, may be involved in the angiogenic response. Degradation prod-

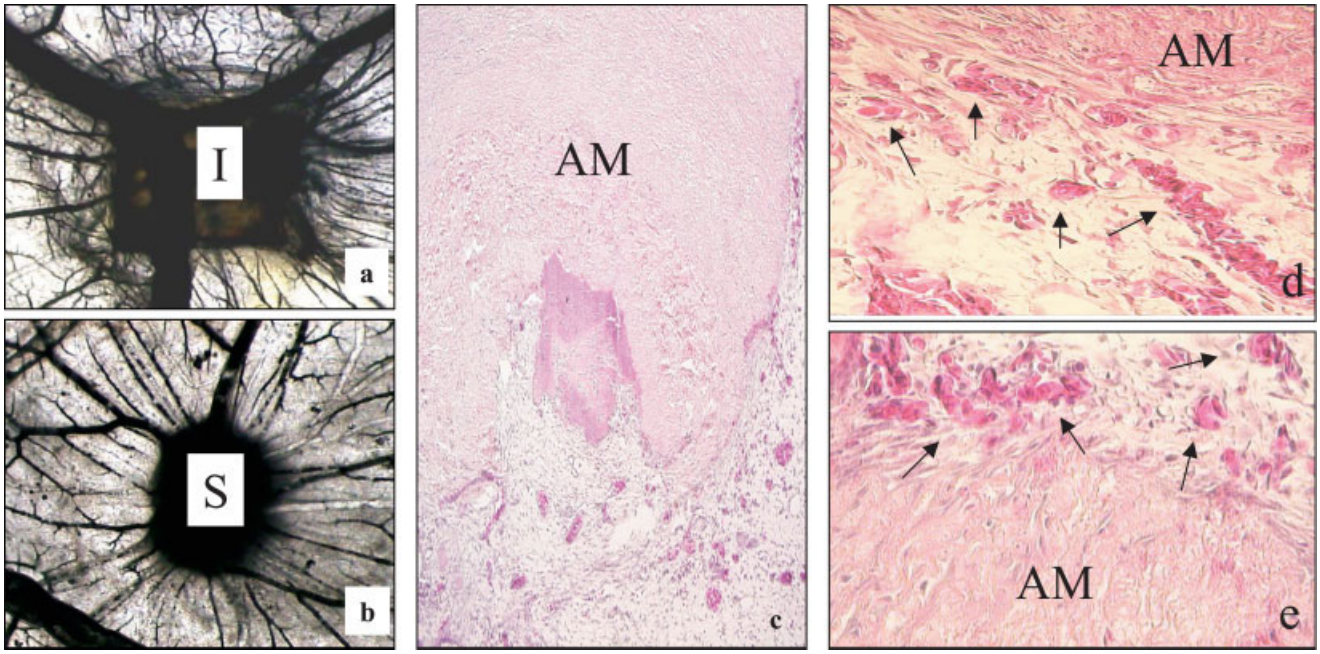

Fig. 2. Macroscopic picture of implant of an aortic acellular matrix surrounded by allantoic vessels developing radially toward it (I) in a spoke-wheel pattern (**a**). The vasoproliferative response is comparable to that induced by a gelatin sponge (S) soaked with FGF-2 (**b**). On a microscopic level, acellular matrix (AM) is adherent to the chorion without invading the mesenchyme (**c**). At higher magnification, numerous newly formed blood vessels (arrows) are located at the interface between the AM and the CAM mesemchyme (**d**). At some points, blood vessels (arrows) are recognizable inside the acellular matrix (**e**). Original magnification: 20× (a, b); 100× (c); 400× (d, e).

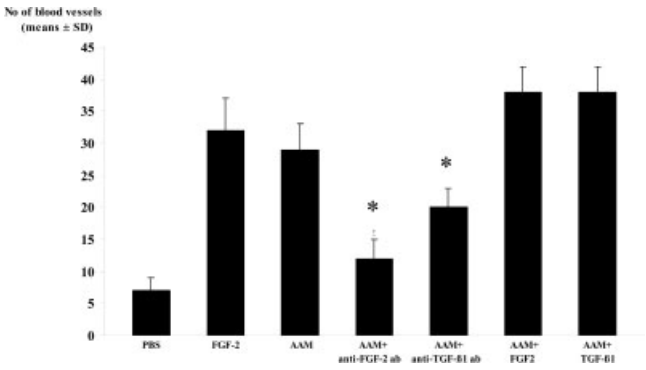

Fig. 3. Macroscopic evaluation of the angiogenic activity of aortic acellular matrices (AAM) in the chick embryo CAM at day 12 of incubation, evaluated as number of blood vessels around the sponges. Asterisk, $P < 0.05$ vs. AAM.

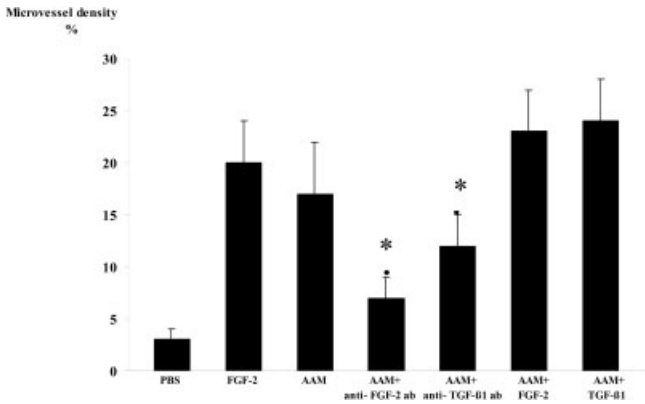

Fig. 4. Microscopic evaluation of the angiogenic activity of aortic acellular matrices (AAM) in the chick embryo CAM at day 12 of incubation, evaluated as microvessel density inside the sponges. Asterisk, P 0.05 vs. AAM.

ucts of elastin, the main component of elastic fibers, induces advential angiogenesis in a rat model (Nackman et al., 1997), while heparan sulfate proteoglycans are involved in the modulation of the neovascularization in different physiological and pathological conditions (Presta et al., 2003) and chondroitin sulfate, the predominant form of proteoglycans in arteries, modulates vascular growth (Tapon-Bretaudiere et al., 2001).

Finally, acellular matrices might induce the release of endogenous angiogenic factors, such as FGF-2 (Ribatti et al., 1995) and TGF-β (Yang and Moses, 1990), stored in the extracellular matrix of the developing CAM. This finding is confirmed by the fact that application of anti-FGF-2 antibody reduced the angiogenic response by 60% while anti-TGF-β antibody by 30%. When the two antibodies were added together, they acted in a synergistic way, reducing the angiogenic response by 90%.

Overall, these data indicate that the aortic acellular matrices induce an angiogenic response in vivo, which may be considered as a consequence of a direct angiogenic activity exerted by the matrices and an indirect induction of the release of angiogenic cytokines stored in the extracellular matrix of the CAM.

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