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Transplantation of human pulpal and gingival fibroblasts attached to synthetic scaffolds

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Autologous tissue grafting for the restoration of oral tissues is limited by several factors, including the availability of sufficient donor tissue. One solution to this problem may be to develop substitute tissue grafts by attaching disaggregated autologous cells propagated *in vitro* to scaffolds composed of natural or synthetic polymers. We have earlier demonstrated that human dental pulp and gingival fibroblasts (HPF, HGF) adhere to non-woven polyglycolic acid (PGA) scaffolds, proliferate and produce extracellular matrix *in vitro*. We now report that such HPF and HGF adhered to PGA scaffolds survive when implanted into subcutaneous sites in immuno-compromised mice. The transplanted cells synthesize and secrete type I collagen, cellular fibronectin and may express genes implicated in transducing bone morphogenetic protein (BMP) signals. Messenger RNA for BMP-2, -4, -7 (OP-1), the BMP type I receptors Act RI, BMPR-1A and 1B, the type II receptor BMPR-II, and type I collagen were detected by reverse transcription-polymerase chain reaction (RT-PCR). These data revealed that three adult human dental pulp and gingival cell populations, each from individual donors, attached to PGA scaffolds and cultured for 24 h *in vitro*, survive implantation and express genes indicative of a capacity to produce extracellular matrix. The implanted cells may also express genes associated with responsiveness to BMP-mediated tissue inductive signals.

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Substantial personal and financial costs attend congenital and acquired loss of oral tissue structure and function, yet few effective therapeutic regimens to restore this loss are available. However, the emerging field of tissue engineering comprises several strategies that address this problem (1). These include the direct delivery of certain genes, proteins or cells to the affected tissues. Restoration of oral tissue structure and function based upon direct delivery of tissue inductive signaling proteins such as growth factors (2–5) and the bone morphogenetic proteins (3, 6–9) is still in development. The therapeutic potential of this approach is substantial but limited by several factors. These include the production of sufficient quantities of biologically active recombinant proteins, the development of suitable carriers for these proteins, the clearance rate of the protein, and the presence of responsive cell populations in or near the tissues to be regenerated. Gene therapy by directly introducing genes, either as naked DNA or recombinant virus, into tissues is also being developed for bone formation (10) but may have similar limitations. Cell-based connective tissue engineering utilizes cultured cell/scaffold constructs developed *in vitro* as connective tissue graft substitutes (1, 11) for specific tissues, including cartilage (12) and bone (13, 14). These studies used cells cultured from the specific tissues, e.g. cartilage and bone marrow, targeted for regeneration.

The ultimate clinical utility of a cell-based approach to oral tissue engineering depends in

part upon the availability and accessibility of autologous cells capable of appropriate differentiation and morphogenesis. For the engineering of oral or dental tissues, autologous cells should be used to avoid immune rejection reactions or immunosupressive therapy. The risk/benefit ratio of immunosupressive therapy for the restoration of oral structure and function is not favorable. Therefore, we seek to determine if cells must be obtained from the specific tissue targeted for repair, or if cells cultured from self-regenerating and more easily biopsied tissues, such as gingiva, can be utilized.

Little is known about the capacity of cells cultured from oral connective tissues such as the gingival corium and pulp to effect tissue repair *in vivo*. Data exist suggesting that mesenchymal tissues contain pluripotential stem cells (11) which are responsive to inductive signals. Bone morphogenetic proteins (BMP) induce reparative dentinogenesis when placed on freshly amputated dental pulps (6, 7), and osteogenesis when placed in fresh extraction sockets (15, 16) and in surgically created tissue pockets in monkey gingival corium (R.B. Rutherford, unpublished observations). This ectopic osteogenic response appeared to be similar to exogenous BMP-induced ectopic bone formation in skin and muscle (17). These data reveal that both dental pulp and the gingival corium contain cells responsive to BMP but respond by producing histologically distinct tissues, reparative dentin or bone, respectively.

To serve as a source of transplantable cells capable of regenerating specific tissues, donor tissues must possess populations of cultivable cells capable of autonomous tissue regeneration and/or of responding to the specific tissue-inductive stimuli of signaling molecules such as BMP. Human pulps, cultured pulp and gingival fibroblasts, grown on standard two-dimensional surfaces, express mRNA for BMPs and their cognate receptors (18). These data demonstrate that cells potentially responsive to BMP signals can be cultured from dental pulp and gingiva.

The overall goal of this research is to develop and test oral cell/scaffold constructs as three-dimensional *in vitro* models of tissue formation and as graft substitutes for the repair or regeneration of specific oral tissues such as bone and dentin. Previous studies demonstrate that HPF/PGA constructs attain, *in vitro*, a cell population density approaching mature human pulp tissue in approximately 45 d (19). Final cellularity is a function of cell proliferation and decreased construct volume; the latter thought to result from PGA dissolution and production of extracellular matrix by the adherent cells. Microscopic inspection suggested that these HPF/PGA constructs were probably too dense to permit sufficient angiogenesis for survival *in vivo*. In contrast human marrow stromal fibroblasts on certain but not all scaffolds tested form bone *in vivo* when implanted immediately after adding the cells to the scaffolds (14). Therefore, we hypothesized that for optimal restoration of oral tissue structure and function, the proportion of cells, extracellular matrix and scaffold will vary among different cells attached to different scaffolds. The experiments reported here initiated testing this hypothesis by studying the capacity of HGF and HPF adhered to nonwoven fibrous PGA scaffolds to survive and function *in vivo* after 24 h incubation *in vitro*.

Material and methods

Human gingival and pulp cells and cell/scaffold constructs

Cultured human cells were obtained by mincing fresh adult dental pulp or gingival biopsies (obtained as surgical waste) from individual donors and explanting the minced tissue as described previously (20). Diploid cell cultures propagated from a single donor (cell strains) were given the designator human pulp or gingival fibroblast (HPF or HGF) and a unique number. The cells of each tested strain were harvested, counted electronically (Coulter Electronics, Hialeah, FL, USA) and either frozen for later use (2nd to 9th passage) or seeded directly onto the scaffolds. PGA scaffolds measuring $1 \times 1 \times 0.3$ cm were cut from sheets of commercially prepared standard nonwoven PGA with fiber diameter of 12 μm, a bulk density of 48 mg/ml at 3 mm thick (Albany International Research, Mansfield, MA, USA) and sterilized by gamma-irradiation. Preliminary experiments determined that 10^6 cells/ml stirred in complete media (DMEM, 10% fetalbovine serum (Hyclone, Provo, UT, USA), penicillin/streptomycin (100 units/100 μg/ml) at approximately 60 rpm at 37°C in a humidified atmosphere of 5% CO_2 and 95% air for 24 h optimized initial cell attachment (B. Buurma, unpublished observations). Initial cellularity was quantified by fluorometric determination of the amount of DNA/scaffold (corrected for differences in scaffold mass). Chemical analyses revealed that the linear range of the fluorometric DNA assay was from 10–500 ng DNA/ml, the lower limit corresponding to 1,500 cells/ml, and that HGF or HPF contained 6.61 ± 1.2 pg DNA/cell; a value consistent with other connective tissue cells (21). PGA had no effect on the DNA assay. Stirring cell suspensions at 10^6cells/ml, 5 ml/cm^2 scaffold for

24 h maximized initial cellularity as measured by DNA content of the HGF/PGA constructs (a mean of $2.9 \times 10^3 \pm 1.4 \times 10^2$ ng/scaffold, $n = 10$). This was independent of cell strain and passage number. The seeded scaffolds were transferred to multi-well dishes, immersed in fresh media, incubated for 24 h at 37°C in a humidified atmosphere of 5% CO_2 and 95% air to remove non-adherent cells. Some scaffolds were developed with HPF or HGF transduced with a recombinant adenovirus, AdRSVntlacZ (Vector Core Lab., University of Michigan), that transduces cells with the bacterial lacZ gene including a nuclear transporter gene (nt) sequence. This gene transports the β-galactosidase into the nucleus where it is detected by enzyme histochemistry. Cells were transduced by incubation with AdRSVntlacZ for 18–24 h at a multiplicity of infection of 100 pfu/cell in media. This results in a transduction efficiency of approximately 50%. Transduced and nontransduced cells were attached to PGA scaffolds as described above. Triplicate constructs comprising 3 strains each of HPF and HGF (transduced and nontranduced) were developed and implanted.

Implantation of cell/scaffold constructs

HPF/PGA, HGF/PGA and PGA control cell/scaffold constructs were implanted into dermal pouches created by blunt dissection laterally from a single dorsal midline incision in immunocompromised mice (N:NIH-bg-nu-xidBR, Charles River, Wilmington, MA, USA). The wounds were clipped to obtain primary closure. The implants were harvested after 3 wk and either immediately fixed by immersion in 4% *p*-formaldehyde or frozen by immersion in liquid nitrogen for RNA extraction. All procedures involving animals were conducted according to protocols approved by the University of Michigan Committee for the Use and Care of Animals.

RNA preparation

Total RNA from cell/scaffold constructs was extracted using the RNAStat-60 method (TEL-TEST B; Friendswood, TX, USA). Frozen cell/scaffold constructs were wrapped with clean aluminum foil, pulverized, and the resultant powder mechanically homogenized in RNAStat-60. The homogenate was mixed with 0.1 volume of chloroform and the phases separated. The RNA was precipitated from the aqueous phase with isopronanol at 1:1 (vol:vol) for 15 min at room temperature or overnight at –20°C, and quantified by UV absorbance spectrometry.

RT-PCR

The S-14, BMP and BMPR primers and procedures for reverse transcriptase-polymerase chain reaction (RT-PCR) utilized in these experiments were the same as described previously. The sizes of the amplfied cDNA fragments range from 571 to 698 bp.

Histology and immunocytochemistry

Histologic and immunocytochemical analyses were performed on constructs fixed by immersion in several volumes of fresh 4% *p*-formaldehyde and embedded in paraffin. Freshly sectioned samples (4–6 μm) were stained alternatively with hematoxylin and eosin, or developed for immunocytochemistry using rabbit anti-human type I collagen or anti-human cellular fibronectin (BioDesign, Kennebunkport, ME, USA) according to the Vectastain ABC and DAB substrate kits (Vector Laboratories, Burlingame, CA, USA). Control sections were reacted with non-immune serum replacing the specific primary antiserum. Staining for lac Z expression was performed on fresh 4% *p*-formaldehyde fixed sections using a commercial kit (Invitrogen, San Diego, CA, USA) according to the manufacturer's instructions.

Results

Triplicate lacZ-transduced and nontransduced HGF and HPF/PGA constructs from three independent cell strains for each cell type (18 total) and 9 control cell-free/PGA constructs were prepared and implanted in immuno-deficient mice. The initial cellularity was kept constant at approximately 3×10^3 ng DNA/cm^2. An equal number of HPF and HGF as cell pellets (unattached to PGA) were also implanted. After 3 wk, ectopic tissue masses were apparent at the sites of implantation for all implants except for the human cell pellets and 67% of the cell-free/PGA scaffolds. Successful implantation was defined as clinically evident grafts that were vascularized and contained transplanted human cells.

By 3 wk, new vessels penetrated all HPF (data not shown) and HGF/PGA implants (Fig. 1A). Residual PGA fibers were visible throughout the implanted tissue. Intact cells appeared in close association with PGA fibers as well as distributed throughout the extracellular matrix that largely filled the interfiber space. To determine if HGF and HPF survived implantation, cells were transiently transduced with a recombinant adenovirus (AdRSVntlacZ) which contains a bacterial β-galactosidase (lacZ) gene. Transduced cells express the lacZ gene product principally in the nucleus by

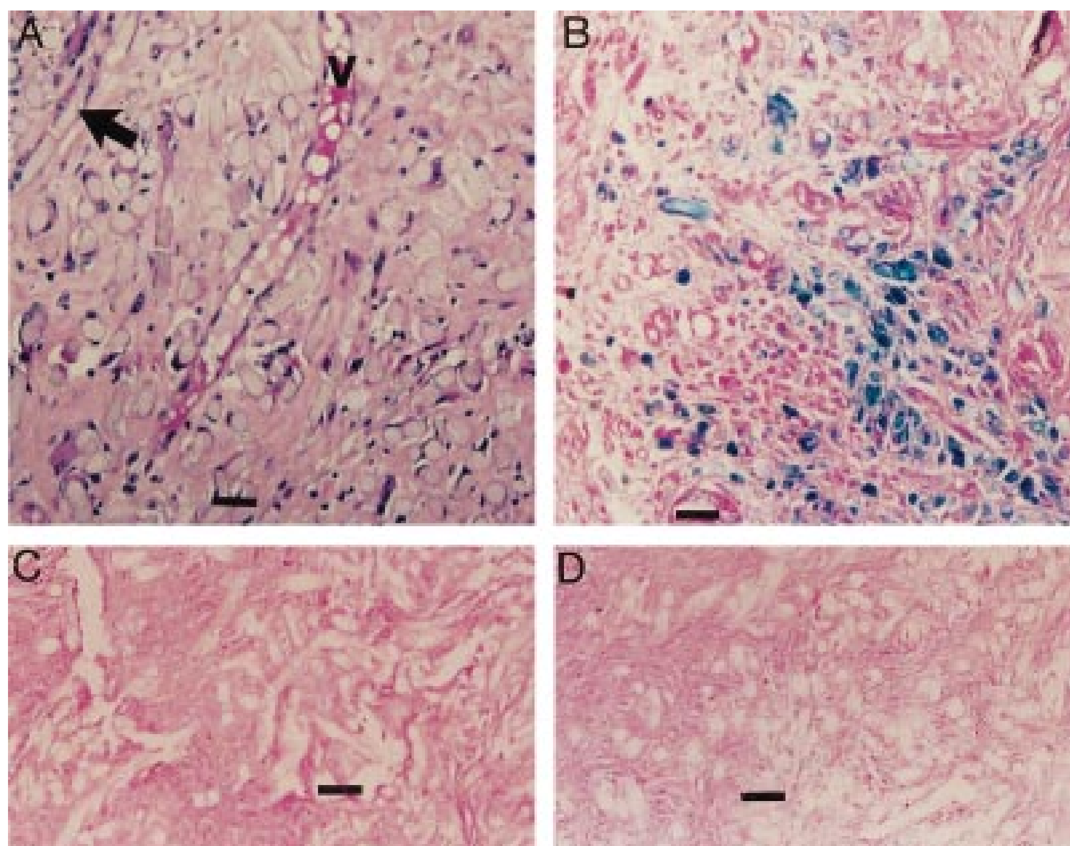

Fig. 1. Photomicrographs of noninfected (A), AdRSVntlacZ HGF/PGA (B) mock-infected (C) and cell-free/PGA (D) constructs after 3 wk *in vivo*. In panel A, cells and extracellular matrix adhered to and interspersed among the PGA fibers (arrowhead) are evident as are vessels (V) that have penetrated the implant. In panel B, cells expresssing the lacZ gene are clearly evident in implants comprised of AdRSVntlacZ transfected HGF cells. No lacZ expressing cells are evident in mock-infected (C) and cell-free PGA control implants (D). Sections stained with H & E stain (A), lacZ detection using X-gal substrate (as described in materials and methods) and counterstained with eosin (B, D). Original magnification ×30, bar equals 25 μm.

virtue of the *nt* gene segment which encodes for a nuclear translocator domain. AdRSVntlacZ infected HPF/PGA, HGF/PGA and PGA control grafts were analyzed for the presence of the transduced lacZ gene. All implants containing *in vitro* infected HGF/PGA (Fig. 1B) or HPF/PGA (data not shown) contained viable transplanted cells as evidenced by expression of the transduced bacterial lacZ gene. Implanted mock-infected HGF/PGA constructs (Fig. 1C) or tissues associated with cell-free PGA scaffolds did not express the lacZ gene (Fig. 1D).

The presence of viable human cells in the implanted tissues was also demonstrated by RT-PCR analyses of total RNA for the presence of human S-14 ribosomal protein specific mRNA (Fig. 2). The PCR primers for human S-14 ribosomal protein, used in these experiments, amplify different sized cDNA fragments from mouse and human cDNA. Total RNA from all of the HGF/PGA constructs cultured for 24 h *in vitro*, implanted and grown for 3 wk *in vivo* contained human and mouse cell S-14 ribosomal protein RNA (Fig. 2). Identical results were obtained from HPF/PGA constructs (data not shown), while the identifiable cell-free PGA implants did not contain human specific mRNA (Fig. 2).

The implanted human cells also appeared to secrete extracellular matrix proteins. Cells

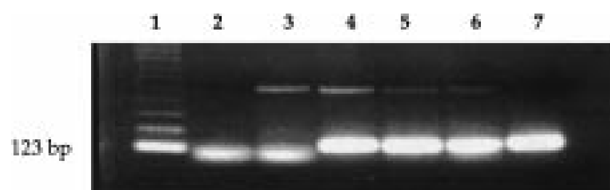

Fig. 2. Implanted HGF/PGA constructs express human S-14 ribosomal protein after 3 wk *in vivo*. cDNA fragments generated using primers, derived from the human gene sequence for S-14 ribosomal protein, in RT-PCR analyses of total RNA are displayed in an enlarged digitized image of an ethidium bromide stained DNA gel. 123-bp fragments are amplified from human RNA; whereas approximately 500-bp fragments are amplified from mouse RNA. Lane contents were as follows: 1) mw markers; 2) control containing no DNA template; 3) cell-free/PGA control; 4) mixture of 7: 1 mouse/human RNA; 5 & 6) HGF/PGA constructs and 7) unimplanted HGF/PGA control construct.

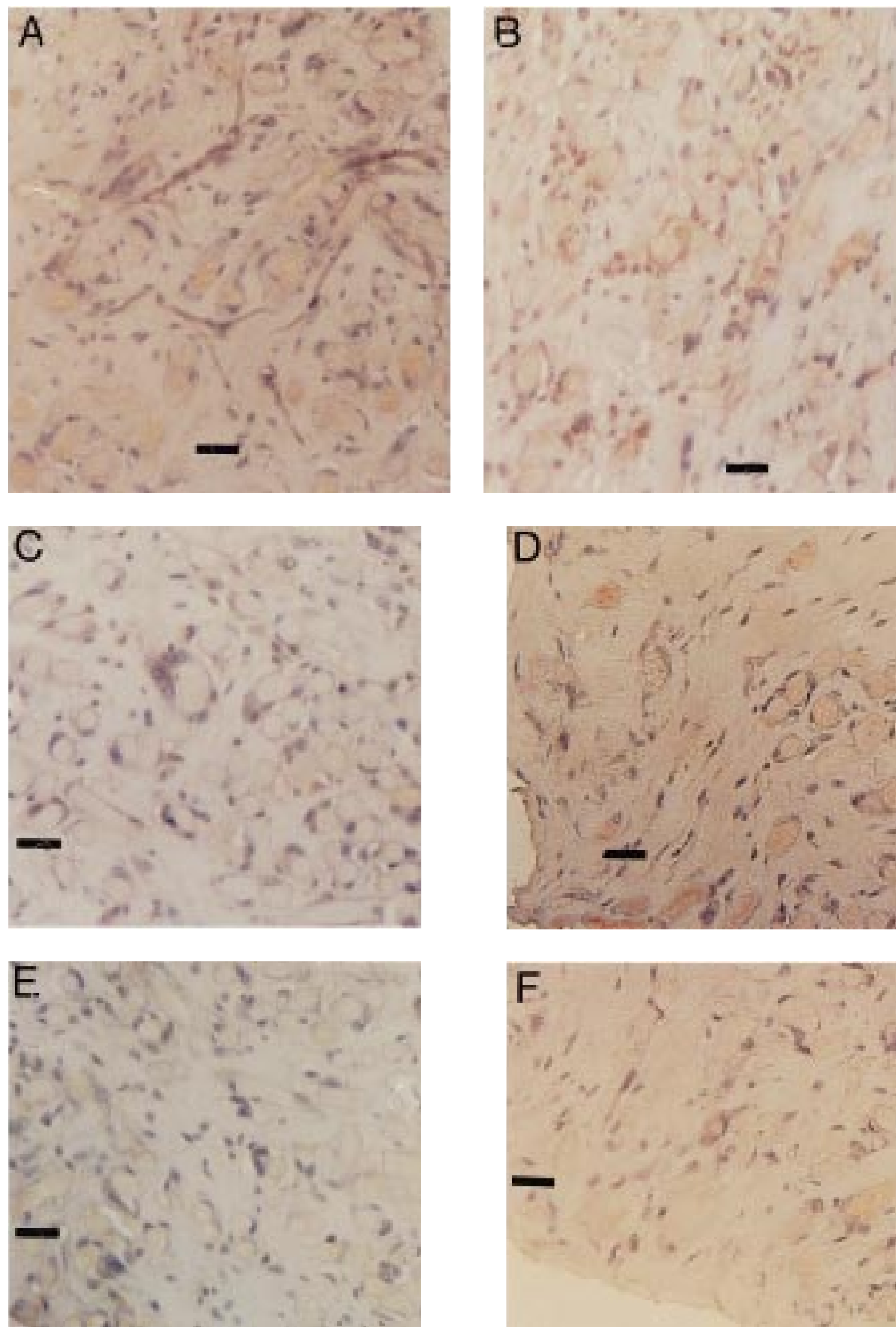

Fig. 3. HGF/PGA implants express human fibronectin and type I collagen after 3 wk *in vivo*. Photomicrographs of HGF/PGA implants developed by immunohistochemistry for human cellular fibronectin (A), and type I collagen (B) after implantation for three weeks are displayed. The presence of human fibronectin and type I collagen coating the PGA fibers and spanning the interfiber spaces is evident in the section developed with specific anti-human antibodies, but not in sections developed with control non-immune sera (C). Human fibronectin (D) or type I collagen (E) is not evident in cell-free/PGA control implants developed with specific antihuman antibodies or nonspecific sera (F). The PGA variably stains in sections developed with both specific and nonspecific antisera. Histologic preparation and immunocytochemistry were performed as described in Material and methods. Original magnification ×30, bar equals 25 μm.

immuno-positive for human cellular fibronectin (Fig. 3A) and type I collagen (Fig. 3B) were adhered to PGA fibers and distributed within the ECM. Equivalent sections developed with non-immune sera were negative (Fig. 3C) as were sections from cell-free PGA control implants analyzed for collagen and fibronectin (Fig. 3D–F). Taken together, these data indicate that human gingival and pulp cells, following attachment to PGA scaffolds and 24 h in culture, survived, synthesized and secreted extracellular matrix proteins for at least 3 wk *in vivo*.

Additional evidence of the survival and function of the implanted human cells is provided by comparison of the expression of several genes associated with responsiveness to bone morphogenetic proteins (BMP). Total RNA from HGF/PGA constructs contained mRNA for BMP-2, -4, -7, BMPR-IA, BMPR-IB, BMPR-II, ActR-I and type I collagen after attachment and incubation for 24 h *in vitro* (data not shown) and after implantation for 3 wk (Fig. 4A). These PCR primers, designed using the human DNA sequences for these genes (GenBank), all routinely amplify similar sized cDNA fragments from mouse and human cDNA except those for BMP-2. These primers routinely fail to amplify a BMP-2 cDNA in RT-PCR analyses of mouse skin total RNA (Gu, unpublished data). The presence of BMP-2 cDNA was not detected in the PGA control (no attached human cells) implants (Fig. 4B) or in control mouse skin samples (data not shown). Similar results were obtained from identical analyses of HPF/PGA total RNA (data not shown). These data further support previous data and reveal that implanted human cells from three non-transduced strains of HGF and HPF attached to PGA and cultured for 24 h *in vitro* survived *in vivo*. These cells may express mRNA for genes required for BMP responsiveness.

Discussion

Cell/scaffold constructs; survival and function

Tissue transplantation is currently limited by the availability of sufficient donor tissue, donor site morbidity as well as the varying effectiveness of available allogenic transplant carrier materials. Recently, significant advances in tissue transplantation have been made by seeding and propagating disaggregated cells on synthetic, polymer scaffolds *in vitro* permitting the development of biological tissue substitutes for implantation *in vivo* (1, 14, 19, 22). These studies demonstrate that variations in cell type, scaffold materials, and *in vitro* development time affect the proportion of cells, extracellular matrix and scaffold in the constructs and the capacity to form tissue *in vivo*. In addition, the capacity of various scaffold materials to support the growth of bone *in vivo* vary (14). This led to our hypothesis that the optimum proportions of cells, extracellular matrix and scaffold for successful implantation vary for each combination.

To initiate testing of this hypothesis, HPF and HGF/PGA constructs, and cultured *in vitro* for 24 h, were implanted *in vivo*. Our data reveal that upon implantation these HGF and HPF/PGA constructs became vascularized and retained viable transplanted cells that synthesized extracellular matrix *in vivo*. Vessels were distributed throughout the mass of the implants, and viable cells were closely associated with the residual PGA fibers and were interspersed within the extracellular matrix (ECM) which largely filled the space among the polymer fibers (Fig. 1). We conclude that these cell populations contain transplanted human cells, since human transplanted cell-specific gene expression was detected at both the protein and RNA levels (Figs. 2, 4). The most compelling evidence is provided by the *in vivo* expression of the transduced lac Z gene in the transplanted tissues. The most plausible explanation for the specific expression of this gene is survival of the transplanted cells that had been transduced with the lac Z gene *in vitro* prior to transplantation. The detection of human S-14 ribsomal protein RNA (Fig. 2) and human proteins associated with extracellular matrix production (Fig. 3) also supports this conclusion. In addition, total RNA from the transplanted tissues contained mRNA for BMP 2, 4 and 7, BMPR as well as type 1 collagen. All of the primers for the human BMP and BMPR genes of interest in this project, except those for BMP-2, hybridize to mouse cDNA sufficiently to support amplification of similarized sized gene fragments. Therefore, the presence of amplified BMP-2 in HGF or HPF/PGA, but not control PGA implants (Fig 4), reinforces the conclusion that the tissues contain surviving transplanted human cells.

It appears from the proportion and distribution

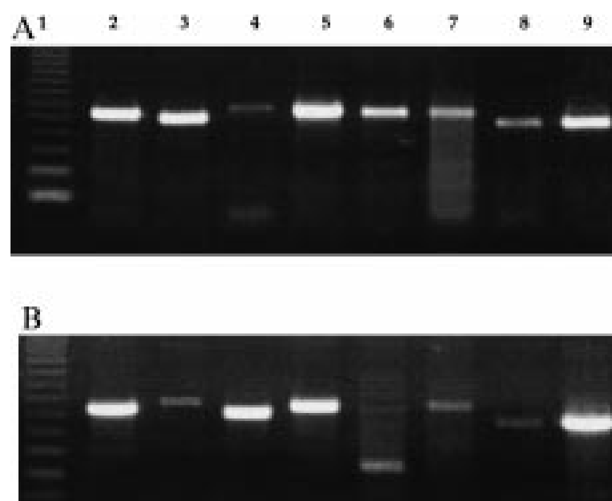

Fig. 4. HGF/PGA constructs express type I collagen, BMP receptor and BMP genes 3 wk after implantation *in vivo.* cDNA fragments generated using primers derived from human gene sequences in RT-PCR analyses of total RNA from HGF/PGA after 3 wk *in vivo* (A) and PGA control constructs after 3 wk *in vivo* (B) are displayed in an enlarged digitized image of an ethidium bromide stained DNA gel. Lane 1, mw markers (123, 246, bp etc.); 2, Act R-I; 3, BMPR-IA; 4, BMPR-IB; 5, BMPR-II; 6, BMP-2; 7. BMP-4; 8, BMP-7; 9, collagen I.

of cells expressing transduced lac Z (Fig. 1) that the bulk of the tissue filling the space within the PGA scaffold is of mouse origin. This result is not surprising since approximately 90% of the volume of the implanted construct is unfilled space at the time of implantation. Mouse granulation tissue is expected to form at a higher initial rate than human tissue. These data demonstrate that HPF and HGF/PGA constructs, developed for only 24 h *in vitro*, survive and secrete extracellular matrix after transplantation. However, it appears likely that more *in vitro* development time is required to increase the cell/extracellular matrix to scaffold ratio to increase proportion of donor tissue in the site of implantation.

Scaffolds

Other experiments have demonstrated that HPF fail to adhere to various hydrogels *in vitro* (23), revealing that not all synthetic polymers are suitable scaffolds for these cells. Krebsbach and co-workers (14) demonstrated that human and mouse marrow stromal fibroblasts (MSF), isolated and passaged in culture, formed bone when dissaggegated, seeded to scaffolds and implanted directly *in vivo.* Interestingly, the osteogenic potential of the human MSF was related to the scaffold; varying from none with demineralized bone powder to strong with a collagen-hydroxyapatite composite (Collagraft Strip™). In parallel experiments, human foreskin fibroblasts formed fibrous connective tissue but not bone *in vivo*. Whereas these studies did not exhaustively test the effects of attached cell number and *in vitro* development time, the data suggest different tissue-forming potentials for distinct cell populations as well as an important role for the type of scaffold utilized as a cell vehicle.

The role of scaffolds is not clear. In our experiments, the presence of a scaffold was necessary since ectopic tissues failed to form at all sites implanted with cells without scaffold. However scaffolding alone is not sufficient to routinely induce tissue formation because 2/3 of the control cell-free PGA implants failed to produce clinically evident tissue masses. Tissue masses containing human cells were present in 18/18 of sites implanted with HGF or HPF/PGA constructs. The reason for the higher frequency of clinically evident tissues found in experimental versus control implants is not apparent at this time.

Scaffolds may facilitate the formation of functional tissues by providing a biodegradable vehicle for the delivery to and retention of organized cell-matrix constructs in a specific *in vivo* site. Synthetic scaffold materials, such as polyglycolic acid, have several potential advantages, including: 1) reproducible synthesis; and 2) control of important mechanical and chemical variables such as shape, size and pore dimensions as well as scaffold composition, and rate of biodegradablity. Polymers that degrade to molecules easily eliminated from the body are also an advantage. Problems inherent with polyacids include the acidification of the environment upon dissolution, however cartilage has been formed *in vivo* from cartilage cell/polyacid constructs (24).

Source of cells for regeneration of specific tissues

The source of cells for seeding the synthetic scaffolds is an important early practical consideration in the development of oral transplant substitutes. In the foreseeable future, cell-based therapy for the regeneration of oral tissues should utilize autologous cells. The risks of immunosuppresive therapy to protect from allograft or xenograft rejection outweigh the benefits of oral tissue regeneration. Ideally, these cells should be easily obtained on an out-patient basis and with minimal donor site morbidity, e.g., a small gingival versus bone marrow biopsy. The tissues should contain cells capable, upon implantation, of producing a functional adult tissue either autonomously, by genetic modification, or in response to appropriate exogenous inductive stimuli (11). Of interest in this regard is the fact that bone morphogenetic proteins (BMP) induce both intra- and extraskeletal bone formation (25) and reparative dentinogenesis when placed on dental pulps (6, 7). In addition, ectopic bone forms when BMP-7 is placed in surgically created connective tissue pockets in monkey gingiva (Rutherford, unpublished observations). This response appeared similar to BMP-induced ectopic bone formation in skin and muscle (17, 26), except that bone formed without a cartilage intermediate. These data suggest that gingival and dental pulp connective tissues both contain cells responsive to BMP but respond by producing histologically distinct tissues, bone and reparative dentin, respectively (27). Previous studies (18) and the data contained herein suggest that populations of cells cultured from these tissue contain cells which express type I and II BMP receptors proposed to mediate BMP inductive signals (for reviews see (28). These data suggest that in the likely event that HPF and HGF/scaffold constructs produce fibrous connective tissue *in vivo*, they contain BMP-responsive populations capable of forming specific mineralized connective tissues upon exposure to the appropriate BMP either in *vitro* or *in vivo*. Experiments testing these ideas are in progress.

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