Hydroxyapatite coating of cellulose sponges attracts bone-marrow-derived stem cells in rat subcutaneous tissue

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The presence of bone-marrow-derived stem cells was investigated in a wound-healing model where subcutaneously implanted cellulose sponges were used to induce granulation tissue formation. When cellulose was coated with hydroxyapatite (HA), the sponges attracted circulating haemopoietic and mesenchymal progenitor cells more efficiently than uncoated cellulose. We hypothesized that the giant cells/macrophages of HA-coated sponges recognize HA as foreign material, phagocyte or hydrolyse it and release calcium ions, which are recognized by the calcium-sensing receptors (CaRs) expressed on many cells including haemopoietic progenitors. Our results showed, indeed, that the HA-coated sponges contained more CaR-positive cells than untreated sponges. The stem cells are, most probably, responsible for the richly vascularized granulation tissue formed in HA-coated sponges. This cell-guiding property of HA-coated cellulose might be useful in clinical situations involving impaired wound repair.

Keywords: wound healing; granulation tissue; cellulose sponge; hydroxyapatite coating; stem cells; calcium-sensing receptor

1. INTRODUCTION

Wound healing is a complex process involving the coordinated interaction of a variety of tissue and cell types. Numerous studies suggest that bone-marrow-derived stem cells participate in tissue repair, e.g. in cutaneous wound healing (Wu *et al.* 2007). Traditionally, bone marrow has been thought to consist of two distinct cell types, namely haemopoietic stem cells (HSCs) and bone marrow stromal cells (MSCs). HSCs differentiate into all mature blood cells whereas MSCs act as a supportive scaffold and a rich source of growth factors to maintain the ideal microenvironment for cells in the bone marrow. During the last few years, the conception of bone marrow stem cells has changed enormously. HSCs are now considered as a heterogeneous cell population that contains not only stem cells committed to haemopoiesis but also endothelial progenitor cells (EPCs; Asahara *et al.* 1999).

Tissue ischaemia and cellular damage caused by trauma release cytokines, which mobilize EPCs from the bone marrow into peripheral blood, and the circulating EPCs then home to sites of nascent neovascularization

and differentiate into mature vascular endothelial cells (Takahashi *et al.* 1999; Kawamoto *et al.* 2003). Besides haemopoietic and vasculogenic plasticity, adult HSCs/EPCs have been shown to possess an ability, at least *in vitro*, to differentiate into non-blood cells, for example to osteoblasts (Long *et al.* 1990; Chen *et al.* 1997; Tondreau *et al.* 2005; Matsumoto *et al.* 2008), cardiomyocytes (Badorff *et al.* 2003; Zhang *et al.* 2004; Iwasaki *et al.* 2006) and even to skeletal, hepatic and neural lineages (Ratajczak *et al.* 2004).

Bone-marrow-derived MSCs are multipotent cells capable of differentiating into numerous cell types, e.g. fibroblasts, cartilage, bone, muscle, brain cells (Sordi *et al.* 2005; Caplan & Dennis 2006; Valtieri & Sorrentino 2008). The MSCs also secrete a large number of growth factors and cytokines that are critical to the repair of injured tissues (Caplan & Dennis 2006). In addition to their multipotency, MSCs have also immunosuppressive effects (Koç *et al.* 2000; Caplan & Dennis 2006; Sasaki *et al.* 2008). Owing to the plasticity of the MSCs, they are widely used in experiments to improve cutaneous wound healing and the results are promising (Yamaguchi *et al.* 2005; Liu *et al.* 2006; McFarlin *et al.* 2006; Cha & Falanga 2007; Kwon *et al.* 2008).

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Wound healing is characterized by the formation of a richly vascularized granulation tissue, which supports the increased nutritional needs of rapidly proliferating and migrating inflammatory and progenitor cells. Implantation of cellulose sponges offers a convenient model to study granulation tissue formation experimentally. The material does not interfere with the normal wound repair process but acts as a chemoattractant for cells that are involved in the process. Cellulose has been used to induce granulation tissue growth not only for research purposes (Mårtson *et al.* 1999; Inkinen *et al.* 2003) but also in clinical studies of wound healing as well (Gay *et al.* 1978; Viljanto *et al.* 1981; Pajulo *et al.* 1999, 2001). In our previous work, we showed that hydroxyapatite (HA) coating of cellulose sponges induces a more effective formation of a highly vascularized granulation tissue than uncoated cellulose when the sponges were implanted subcutaneously in rat (Tommila *et al.* 2008). The HA coating was formed by treating cellulose with a bioactive glass, which creates a silica-rich calcium phosphate layer (Ekholm *et al.* 2005). In the bone marrow, the HSC niche is located close to the endosteal surface and is regulated via calcium-sensing receptors (CaRs) of HSCs (Adams *et al.* 2006; Drüeke 2006). The HA coating resembles the natural mineral composition of bone (Ekholm *et al.* 2005) and might therefore attract bone-marrow-derived stem cells. Thus, we wanted to investigate whether the granulation tissue-stimulating ability of the HA-coated cellulose implant is due to increased homing of bone-marrow-derived stem cells.

2. MATERIAL AND METHODS

2.1. Treatment of cellulose sponges with bioactive glass

HA-coated cellulose was prepared as described previously (Ekholm *et al.* 2005). In short, simulated body fluids (SBF) with ion concentration close to 1 and 1.5 times that of human blood plasma were prepared, buffered to pH 7.4 and sterilized by filtering. The initial calcium phosphate layer was precipitated on regenerated, oxidized cellulose sponges (Vivoxid/Cellomeda Ltd, Finland) by the biomimetic method of Kokubo *et al.* (1991). Mineralization was initiated in 500 ml 1×SBF supplemented with 2 g of a specific granular bioactive glass (S53P4, Vivoxid Ltd) at 37°C for 24 hours and was then grown in 500 ml 1.5×SBF at the same temperature under continuous shaking. After 14 days, the sponges were washed briefly with sterile water, dried at room temperature in a laminar hood and the presence and homogeneity of the mineral layer were confirmed by scanning electron microscopy.

2.2. Test animals and surgical procedures

Mineralized and untreated celluloses were cut into pieces of 5×5×10 mm, autoclaved and implanted into nine male Sprague–Dawley rats (mean weight 304±17 g, age 10–13 weeks). The animals were anaesthetized with a subcutaneous injection of a mixture of midazolam (Dormicum, Roche, Switzerland) and fentanyl–fluanisole

(Hypnorm, Jansen Pharmaceuticals, Belgium) in sterile water (1 : 1 : 2). The backs of the animals were shaved, disinfected with a 5 mg ml⁻¹ solution of chlorhexidine gluconate (Klorhexol, Leiras, Finland) and draped with sterile towels. Two separate 2 cm long midline incisions, cranial and caudal, were made on the back of the animals. The sterilized sponge implants were moistened with 0.9 per cent NaCl solution and inserted bilaterally into the pockets of the subcutaneous space without fixation. Mineralized and non-mineralized implants were inserted alternately into the left and right sides to reduce biological variation. Skin wounds were closed with interrupted absorbable sutures (4-0 Vicryl-rapid, Ethicon).

The rats were allowed to recover from anaesthesia in the operation room under a heating lamp and were housed individually in the cages with free access to food pellets and water. The animals were killed via cervical dislocation under CO₂ anaesthesia: three animals at day 3 and six animals at day 7 post-operatively. The implants were dissected free from the surrounding connective tissue. All implants from day 3 and the implants from three animals from day 7 were fixed in 4 per cent phosphate buffered paraformaldehyde for 16 hours for histology. The implants from the remaining three rats at day 7 were used in cell-sorting analysis.

The animal facilities are managed according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes and inspected for GLP Compliance. The study design was approved by the Animals Ethical Committee of the State Provincial Office of Southern Finland.

2.3. General histological and immunological stainings

The fixed sponge samples were embedded in paraffin and sectioned longitudinally with a microtome. The sections were stained with haematoxylin–eosin (HE) and Giemsa for light microscopy examination.

Immunostainings of formalin-fixed paraffin-embedded sections were carried out as described previously (Tommila *et al.* 2008) using the avidin–biotin–peroxidase complex technique (Vectastain ABC kit, Vector Laboratories Inc., USA). The antibodies used in this work as stem cell markers were CD34 (rat monoclonal; Santa Cruz Biotechnology, USA), c-kit (CD117; rabbit polyclonal; Santa Cruz Biotechnology) and stro-1 (mouse monoclonal; Zymed Laboratories, USA). All primary antibodies were diluted 1 : 50. CD34 is the most commonly used HSC marker, whereas c-kit is generally found in many premature cell lineages. Stro-1, notably, has a superior position as a mesenchymal stem cell marker. For counter-staining, the sections were kept in Papanicolaou's solution 1a, Harris' haematoxylin solution (Merck, Germany) for 30 s.

CaR was visualized by immunofluorescence using an anti-CaR mouse monoclonal antibody (1 : 50; Santa Cruz Biotechnology). Texas-Red-labelled goat anti-mouse IgG (Molecular Probes, USA) was used as a secondary antibody. Nuclei were counter-stained with 4', 6-diamidino-2-phenylindole (Molecular Probes).

Figure 1. Histological stainings of cellulose implants show more cells in the HA-coated sponges. The invading cells are gathered around the cellulose fragments (cf): (i) coated, (ii) uncoated. Giemsa-stained (a) 3- and (b) 7-day-old implants (scale bar, 25 μm). (c) Giemsa-stained whole implants at post-operative day 7 (scale bar, 1000 μm). (d) HE-stained sections of 7-day-old implants (scale bar, 50 μm).

2.4. Cell detaching and flow cytometric measurements

Cells from 7-day-old implants of three rats were detached enzymatically. Immediately after removal,

the implants were placed on ice in Falcon tubes with 2 ml of α -minimum Eagle's medium (α -MEM; Gibco, USA) complemented with 15 per cent foetal bovine serum (Biochrom AB, Germany). The implants were

cut into small pieces on a Petri dish in a laminar flow hood and placed into new Falcon tubes, and 2 ml of prewarmed (37°C) enzyme mixture (0.1% collagenase (Boehringer Mannheim, Germany) and 0.05% trypsin (Sigma, USA) in sterile phosphate-buffered saline) was pipetted on top. The tubes were gently shaken for 45 min at 37°C, and then the samples were filtered through nylon membranes with 2 ml of α -MEM. The cell mixture was centrifuged (1600g, 4 min) and the supernatant was removed. The cells were washed twice with 2 ml of α -MEM and finally suspended in 1 ml of α -MEM and used for flow cytometry.

Flow cytometric measurements were done with a FACSCalibur instrument (Becton Dickinson, USA) and data analysis was performed with CYFLOGIC software (Cyflo, Finland). The living cells were gated from forward-scatter/side-scatter dot plot. The proportion of c-kit-positive cells was measured from the samples. Alexa Fluor 488-conjugated goat anti-rabbit IgG (Molecular Probe) was used as the secondary antibody to detect anti-c-kit (CD117; rabbit polyclonal, Santa Cruz Biotechnology).

The mean fluorescence intensity of HA-coated samples versus uncoated controls was compared using two-tailed *t*-test. Values are presented as mean $\pm$ s.d. A value of $p < 0.05$ was considered significant.

3. RESULTS

3.1. Visual inspection showed more cells and granulation tissue in HA-coated implants

As we reported earlier (Tommila *et al.* 2008), granulation tissue grew notably faster into the HA sponges compared with uncoated sponges and filled the HA-coated sponges within two weeks. This phenomenon was also evident in the Giemsa-stained sections, in which the staining was more intense in HA sponges both at days 3 and 7 (figure 1*a–c*). The foreign body reaction started from the sponge surfaces from where the cells invaded further towards the sponge centres. The cells in the untreated control sponges appeared similar to the cells in the HA-coated sponges, but were in smaller quantity (figure 1*d*).

3.2. Flow cytometric analysis revealed more c-kit-positive cells in HA-coated sponges

Enzymatically detached cells from 7-day-old implants were screened for the expression of c-kit, a common premature cell marker. There was a significantly ($p < 0.05$) higher c-kit expression in HA-coated sponges than in the uncoated ones (figure 2).

3.3. Common stem cell markers stained more cells in HA-coated implants

All stem cell markers used in this study stained numerous cells in the HA sponges, whereas hardly any positive cells were observed in the controls (figure 3). The stainings of c-kit and CD34 were emphasized in the sponge centre, while the common mesenchymal stem cell marker, stro-1, stained cells

Figure 2. Flow cytometry analysis revealed more c-kit-positive cells in HA-coated 7-day-old implants. (a) Histogram showing the expression of c-kit-positive cells extracted from the cellulose implants. The filled histogram under the segment of a line shows the intensity of c-kit-stained cells, whereas the curve indicated by arrows shows the background stained only with secondary antibody ((i) coated and (ii) uncoated). (b) The HA-coated implants contained significantly more c-kit-positive cells than untreated controls ($p < 0.05$, column represents mean $\pm$ s.d.).

closer to the sponge surfaces, i.e. the area that was rich in collagen-containing granulation tissue. All studied stem cells, especially in the HA-coated implants, were located in close contact with cellulose fragments.

3.4. HA-coated sponges contained more CaR-positive cells

The CaR is expressed in many cell types, including macrophages and monocytes. These receptors have also proven to be important in the HSC storage in bone marrow (Drüeke 2006). An abundant amount of CaR-positive cells was observed in HA-coated samples not only in the new granulation tissue, but also in the central area of the implants. Most of the CaR-positive cells were gathered closely to the HA-coated cellulose fragments (figure 4). Similar cells were seen in the plain cellulose sponges, but in remarkably lesser amounts and mainly near the implant surfaces.

4. DISCUSSION

At a wound site, local and infiltrated cells release chemokines, which recruit blood-circulating stem and progenitor cells. These chemokines also increase bone marrow cell mobility, thus facilitating cell mobilization into the peripheral blood and into the sites of wound healing (Sasaki *et al.* 2008). When implanted subcutaneously in the rat, the HA-coated cellulose sponges induced a stronger and quicker inflammatory response than untreated sponges. The HA layer augmented the recruitment of macrophages, which provide a continuing source of growth factors and cytokines that affect the local environment (Tommila *et al.* 2008).

Figure 3. Stem cell markers in 7-day-old implants ((i) coated and (ii) uncoated). (a) Numerous c-kit-positive cells were observed in the sponge centre of HA-coated implants. (b) Anti-CD34-stained small rounded cells in the middle of the HA-coated sponge. (c) Stro-1, recognizing mesenchymal progenitors, stained cells closer to the sponge surface. Arrowheads point at positively stained (brownish coloured) cells. Scale bar, 25 µm.

Figure 4. Calcium-sensing receptor-positive cells in 7-day-old implants. (a) CaR-positive cells in the centre of the HA-coated sponge are accumulated in clusters near cellulose fragments. (b) Only a few CaR-positive cells can be seen in the control. (i) Lower magnification (10× objective lens) of the same section and (ii) nuclear stainings. Scale bar, 25 µm.

In the present study, we showed that the HA coating of cellulose sponges also increased the homing of bone-marrow-derived stem cells. The implants were screened for c-kit expression by flow cytometry, and a significantly higher expression was observed in HA-coated samples. Different types of stem cells were further identified by immunohistochemistry. C-kit- and CD34-positive cells indicated that HSCs were the first

ones to invade the empty centres of the sponges, while MSCs (stro-1-positive) were found in the forming granulation tissue.

In the bone marrow, undifferentiated HSCs are detected near the endosteum, the thin layer of cell-rich connective tissue that lines the medullary cavity of long bones, in the so-called endosteal stem cell niche (Haylock & Nilsson 2005). At this site, the bone is in a

constant turnover; bone is laid down by osteoblasts and removed by specific macrophages, the osteoclasts. Owing to bone degradation, soluble calcium ions (Ca^{2+}) are released in the bone marrow fluid. Cells, including haemopoietic progenitors as well as monocytes/macrophages, respond to extracellular ionic calcium concentrations through a calcium-sensing G-protein-coupled receptor, CaR. Therefore, CaR seems to have a function of retaining HSCs in close physical nearness to the endosteal surface (Adams *et al.* 2006). The HA coating used in this study resembles the HA of bone (Ekholm *et al.* 2005). Plentiful giant cells/macrophages are gathered around the mineralized cellulose (Tommila *et al.* 2008). We hypothesized that while macrophages try to phagocyte or hydrolyse the silica-rich foreign material, Ca^{2+} is released, which creates a beneficial milieu for the gathering of circulating haemopoietic progenitors. Indeed, CaR-positive primitive cells were observed near the mineralized cellulose fibres, supporting our theory that circulating haemopoietic progenitors home to the mineralized cellulose and differentiate into various haemopoietic cells.

According to our microarray data of an ongoing study to further characterize gene expression in granulation tissue (unpublished data), cells in the granulation tissue in the cellulose sponges express stromal-derived factor-1 (SDF-1), a vital chemokine for stem and progenitor cell recruitment in tissue repair after injury (Kollet *et al.* 2003; Ratajczak *et al.* 2004; Kaplan *et al.* 2007; Schantz *et al.* 2007). The microarray data also revealed that the expression of CXCR4, the receptor for SDF-1, is significantly upregulated in HA-coated sponges, indicating more stem and progenitor cells in these sponges. The homing mechanisms that guide the recruitment of MSCs are not yet fully understood, but SDF-1/CXCR4 is described to be an important part of the homing process (Wynn *et al.* 2004; Schantz *et al.* 2007).

In the bone marrow microenvironment, there seems to be an important functional dialogue between HSCs/EPs and MSCs (Satoh *et al.* 1997; Valtieri & Sorrentino 2008), but their exact roles in tissue repair remain unclear. It is likely that both cell types are needed in wound healing. MSCs express transcripts encoding proteins that regulate a broad range of biological activities. They have a capacity to effect tissue repair via multiple mechanisms including promoting the survival and proliferation of endogenous cells, inhibiting inflammatory and immune responses (Phinney & Prockop 2007). MSCs release factors that not only enhance the regeneration of non-haemopoietic tissues, but also yield proangiogenic factors essential for HSCs/EPs survival, proliferation and differentiation (Kinnaird *et al.* 2004; Honczarenko *et al.* 2006), thus having a role in blood vessel formation as well. Wounds receiving implanted MSCs also show enhanced angiogenesis (Chen *et al.* 2008), which further indicates their importance during wound repair.

Recovery of blood flow at an injury site is considered to be a prerequisite for tissue reconstitution. Vasculogenesis, the de novo process by which progenitor stem cells differentiate and give rise to a replacement

vascular network, was earlier believed to take place only during embryonic development. Bone-marrow-derived endothelial progenitors have, however, been identified in the peripheral blood and shown to participate in post-natal new blood vessel formation (Velazquez 2007). The granulation tissue of the wound is, thus, vascularized by the processes of angiogenesis or vasculogenesis, or both.

Here, we noted that the HA-coated sponges had an increased number of cells positive for c-kit and CD34, both known to be markers for endothelial lineage cells, and more stro-1-positive cells indicating more abundant MSCs. The more vascularized granulation tissue in HA-coated sponges (Tommila *et al.* 2008) might be a result of more abundant homing of bone-marrow-derived stem cells to HA-coated cellulose.

To conclude, HA-coated cellulose sponges used to induce and harvest granulation tissue seem to have cell-guiding features that facilitate homing of bone-marrow-derived stem and progenitor cells more efficiently than untreated cellulose. This property of HA-coated cellulose together with its capacity to promote proliferation of richly vascularized connective tissue (Tommila *et al.* 2008) might have potential clinical application in the treatment of poorly healing wounds.

The study design was approved by the Animals Ethical Committee of the State Provincial Office of Southern Finland.

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