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Original Article

Histological characterization of bone marrow in ectopic bone, induced by devitalized Saos-2 human osteosarcoma cells

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Abstract: Devitalized Saos-2, cultured human osteosarcoma cells, or guanidinium-hydrochloride (GuHCl) extracts of these cells, induce ectopic bone and marrow formation when implanted subcutaneously in Nu/Nu mice. The aim of the present study was to characterize the bone marrow induced by Saos-2 cell extracts, specifically to determine which of the four major hematopoietic cell lineages: erythropoietic, granulopoietic, lymphopoietic and megakaryocytic, are induced by Saos-2 cell derivatives. Methods: Immunohistochemical localization of specific antigens was used to determine the presence of each major cell type (glycophorin A for erythropoietic, neutrophil elastase for granulopoietic, factor-VIII related antigen for megakaryocytes, and CD79a for B lymphocytes). Results: Standard H & E stains confirmed the presence of normally organized apparently complete bone marrow within all newly induced bone at 3 weeks post-implantation of devitalized Saos-2 cells. Immunohistochemistry confirmed the presence of erythropoietic cells, granulopoietic cells, megakaryocytes and B lymphocytes in the ectopic marrow. Conclusion: Saos-2 cells (freeze-dried) or their extracts, implanted subcutaneously into Nu/Nu mice, can induce normal marrow that is host-derived, and contains all major hematopoietic cell lineages. Clinical Significance: Saos-2 induced marrow could potentially restore deficient marrow and promote bone repair.

Keywords: Bone marrow induction, bone tumors, hematopoiesis, lineage-specific biomarkers, osteosarcoma

Introduction

It is known that the subcutaneous induction of ectopic bone in vivo, can lead to the formation of hematopoietic marrow within the induced ossicle [1-3]. Agents known to induce new bone with hematopoietic marrow include: 1) decalcified bone matrix [4, 5], 2) extract of cultured Saos-2 human osteosarcoma cells [6], and 3) bovine bone morphogenetic proteins (BMP) [7]. In all of the above examples, new bone forms by a process of endochondral bone formation. Marrow makes its first appearance at about 12 to 16 days after implantation of the bone inducing agent, within sinusoidal cavities of the newly formed ossicles [4]. In the current study, it is our hypothesis that in the newly formed marrow, cells of the erythrocytic, granulopoietic, and megakaryocytic lineages can be detected histologically by immunoexpression of lineage-specific biomarkers.

Saos-2 human osteosarcoma cells are a source of extractable bone-inducing agent [6]. Bone can be induced by placing collagen implants of devitalized (freeze-dried) Saos-2 cells or GuHCl extracts of Saos-2 cells subcutaneously in 2-3 weeks old Nu/Nu mice [6]. The SaOS-2 implant induces endochondral bone formation, with cartilage appearing at approximately one week. Cartilage is replaced by new bone and marrow at 12 days to 2 weeks post-implantation [2]. Hematopoietic marrow appears between endosteal bone trabeculae of the Saos-2 cell-induced ossicle [6].

Saos-2 cells express, synthesize and secrete a combination of bone morphogenetic proteins (BMPs), plus other proteins that apparently interact to induce ectopic bone and marrow [2]. Semi-purified Saos-2 cell extracts also have been shown to augment healing and osseous union of large, normally non-united femoral defects in rats [8].

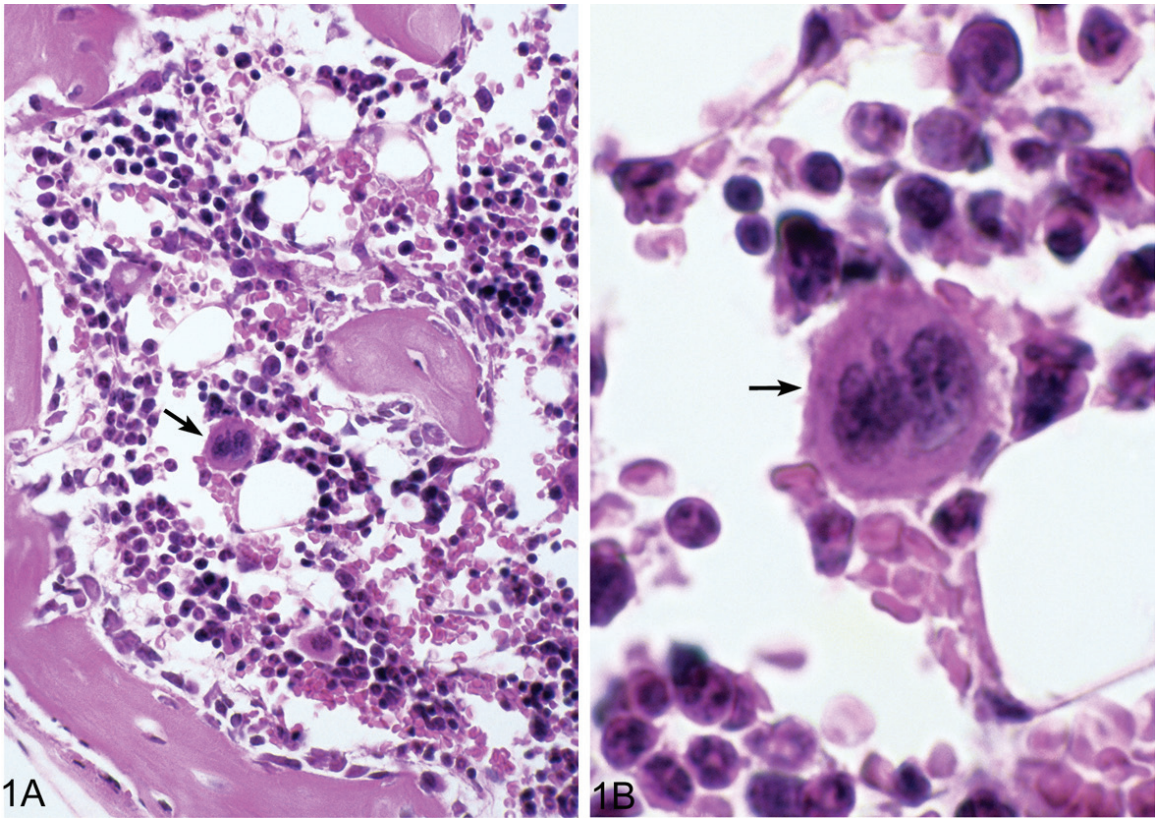

Figure 1. Microscopic images of Saos-2 induced bone and marrow, formed at 3 weeks post implantation of devitalized Saos-2 cells into Nu/Nu mouse. 1A. A cortex-like trabeculum of new bone (lower left) surrounds an inclusion of normal appearing marrow, containing a megakaryocyte (arrow) (Hematoxylin and Eosin, microscopic magnification 400 X). 1B. The megakaryocyte depicted in Figure 1A (arrow) is shown here in greater detail (1000 X).

Saos-2 cell induced marrow has never been fully characterized, and it has not yet been determined whether all four major hematopoietic cell lines i.e erythropoietic, granulopoietic, lymphopoietic, and megakaryocytic cell lineages are generated during Saos-2 cell bone induction. The objective of this study was, to use specific immunohistochemical markers to identify and quantitate hematopoietic cells of erythropoietic, granulopoietic, lymphopoietic and megakaryocytic lineage in the ectopic marrow induced by devitalized Saos-2 cells or their extracts.

Materials and methods

Preparation of implants

Saos-2 derived implants were made from either 10 mg of freeze-dried Saos-2 cells or 3 mg of microsome-sized Saos-2 cell fragments, retained by a 0.45 micron Millipore filter (retentate) and extracted into 6M urea [3]. Urea was removed from retentate fractions by dialysis prior to implant preparation. Two milligrams of bovine collagen (Cohesion, Palo Alto, CA) was mixed with freeze-dried cells or retentate. The pellets were implanted subcutaneously adjacent to the *latissimus dorsi* muscles of anesthetized Nu/Nu mice (Charles River Laboratories, Boston, MA, USA) and the skin was closed by sterile stainless steel staples [6]. This was done with authorization of the University of Kansas Medical Center (KUMC) Institutional Animal Care and Use Committee.

Preparation of ectopic (induced) and tibial (normal) bone sections of mice

Fourteen days, post-implantation of devitalized Saos-2 collagen implants in Nu/Nu mice, all animals were anesthetized and euthanized by cervical dislocation, and newly formed ossicles were removed surgically. Normal tibial (unimplanted; control) bone and marrow were collected in biopsies from 5 weeks-old normal

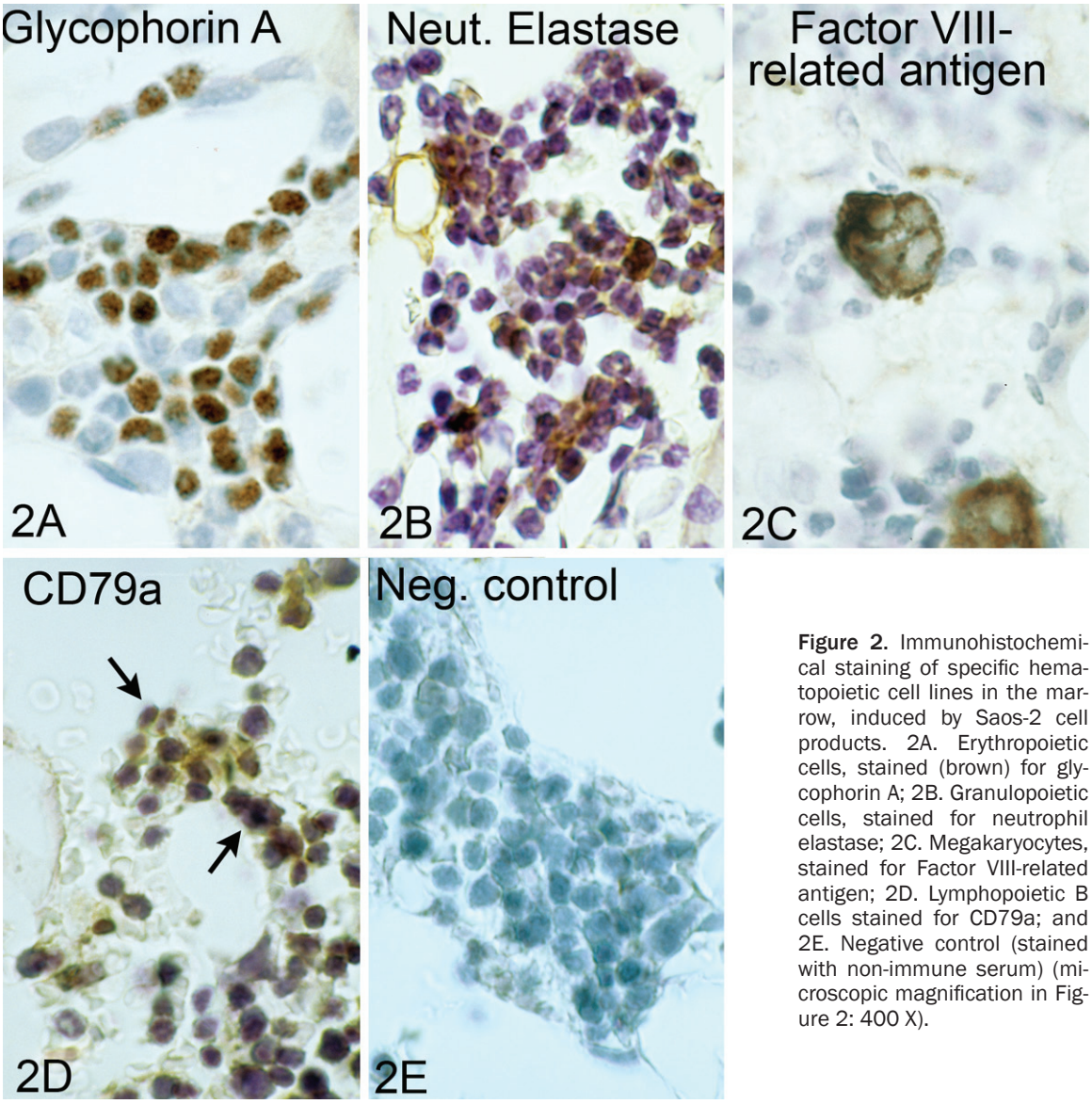

Figure 2. Immunohistochemical staining of specific hematopoietic cell lines in the marrow, induced by Saos-2 cell products. 2A. Erythropoietic cells, stained (brown) for glycophorin A; 2B. Granulopoietic cells, stained for neutrophil elastase; 2C. Megakaryocytes, stained for Factor VIII-related antigen; 2D. Lymphopoietic B cells stained for CD79a; and 2E. Negative control (stained with non-immune serum) (microscopic magnification in Figure 2: 400 X).

mice. Ectopic bone and normal tibias were fixed for 24 h in 4% paraformaldehyde and decalcified in 10% ethylene diamine tetra-acetic acid (EDTA) for up to 10 days and embedded in paraffin and sectioned.

Histological staining for identification of bone marrow cell types

Five micron-thick, decalcified sections of implant-induced bone with marrow, and control tibial bone marrow sections were stained by conventional histological Hematoxylin and Eosin (H&E) staining, and evaluated by light microscopy for bone marrow architecture and lineage specific cell types.

Immunohistochemistry

Immunohistochemical staining was performed according to the labeled streptavidin biotin (LSAB) method using a DAKO LSAB kit (DAKO, Carpinteria, CA, USA). Mouse monoclonal anti-human glycophorin A (Dako, Carpinteria, CA) and monoclonal rat anti-mouse TER-119 (BD Biosciences, Franklin Lakes, NJ) were used to detect erythropoietic cells. Rabbit polyclonal anti-human Factor VIII-related antigen from Dako was used to detect megakaryocytes. Rabbit polyclonal anti-human neutrophil elastase (Santa Cruz Biotech, CA) was used to detect granulopoietic cells and mouse monoclonal anti-human CD79a antibody (Vantana,

Table 1. Total Cell counts in marrow of ectopic bone

Post-implantation age of Saos-2 induced ossicles	Total cell count/ high power field (HPF)
2 weeks	471
3 weeks	540

Tucson, Arizona) was used to stain for B lymphopoietic cells. Primary antibodies were used at the following concentrations: anti-human glycophorin A (1:500), anti-human neutrophil elastase (1:20), CD79a (1:50) and Factor VIII-related antigen (prediluted form as supplied from the company).

Number of cells in ectopic bone marrow

Hematoxylin and Eosin stained slides of two and three week old ectopic ossicles, were used to count total number of marrow cells using a light microscope at a magnification of 400X. Total numbers of cells were recorded from an average of five, 400X microscopic fields. Each of the five randomly selected individual fields was also examined for the presence of erythropoietic, granulopoietic, lymphopoietic and megakaryocytic cells. The results were expressed as the number of cells per high–power field [1, 9].

Results

A well-organized and apparently complete bone marrow, as indicated by the presence of all four major hematopoietic cell types was demonstrated in the ectopic bone (**Figure 1A** & **1B**) by conventional histochemical H and E staining. Immunohistochemistry confirmed the presence of erythropoietic cells, granulopoietic cells, megakaryocytes and B lymphocytes (**Figure 2A-E**) based on the immunoexpression of lineage-specific biomarkers i.e. glycophorin A and TER119 (data not shown) for erythropoiesis; neutrophil elastase for granulopoiesis; CD79a for B lymphocytes and Factor VIII for megakaryocytes. Total marrow cell counts showed more cells in the three week-old ossicles than in 2 week-old (**Table 1**). Percentage of four major bone marrow cell types in three week old ectopic marrow is shown in **Table 2**.

Discussion

In this study, conventional H and E staining of ectopic bone showed typical morphological features of different major cell-types (erythropoietic, granulopoietic, megakaryocytic, lymphocytic cells), thereby indicating the presence of a histologically complete induced marrow within the newly formed ossicles. The presence of erythropoietic, granulopoietic, megakaryocytic and B lymphopoietic cells was clearly confirmed by immunostaining.

The total ectopic marrow cell count was greater in three-week old marrow. The distribution (%) of specific cell lineages in the ectopic marrow generated 3 weeks post-implantation of collagen matrices containing devitalized SaOS-2 cells, as shown in **Table 2** suggests that the cellular composition of Saos-2 induced marrow is approximately that of normal marrow. This finding suggests that, freeze dried Saos-2 cells when injected intramuscularly into Nu/Nu mice [6], have the potential to promote ectopic marrow formation in human recipients. Clearly, this is a good model to study the induction of hematopoiesis.

The mechanism by which Saos-2 cell products can induce ectopic bone and marrow is not fully understood. It appears that Saos-2 cell products, like demineralized bone matrix (DBM) [5] and isolated bone morphogenetic proteins [7], can induce endochondral bone which then serves as an osteoblast-lined “niche” for the generation of hematopoietic marrow. Recent data has shown that osteoblasts support hematopoiesis by providing a supportive microenvironment in which hematopoietic stem cells can establish growing colonies of new marrow cells after initial bone development [10]. The mechanism by which osteoblasts can provide such a niche for hematopoietic cells is still not completely understood, although there is evidence that hematopoiesis is augmented by the expression of granulocytic colony stimulating factor (CSF) [11], PTH receptors [12], and BMPs [13]. Normal hematopoiesis requires a balance between hematopoietic growth factors and myelosuppressive factors for generation of optimum number of cells for specific lineages.

The site of origin of the new, ectopic marrow hematopoietic stem cell (HSC) precursors could

Table 2. Percentage of four major cell types in three week-old ectopic marrow

Hematopoietic lineage-specific cell types	Average number of lineage specific cells (Cells in five different area in HPF)	% of specific cells
Erythropoietic cells	1279/2510	51%
Granulopoietic cells	471/2818	17%
B-lymphocytes	261/ 2740	10%
Megakaryocytes	6/2628	0.002%

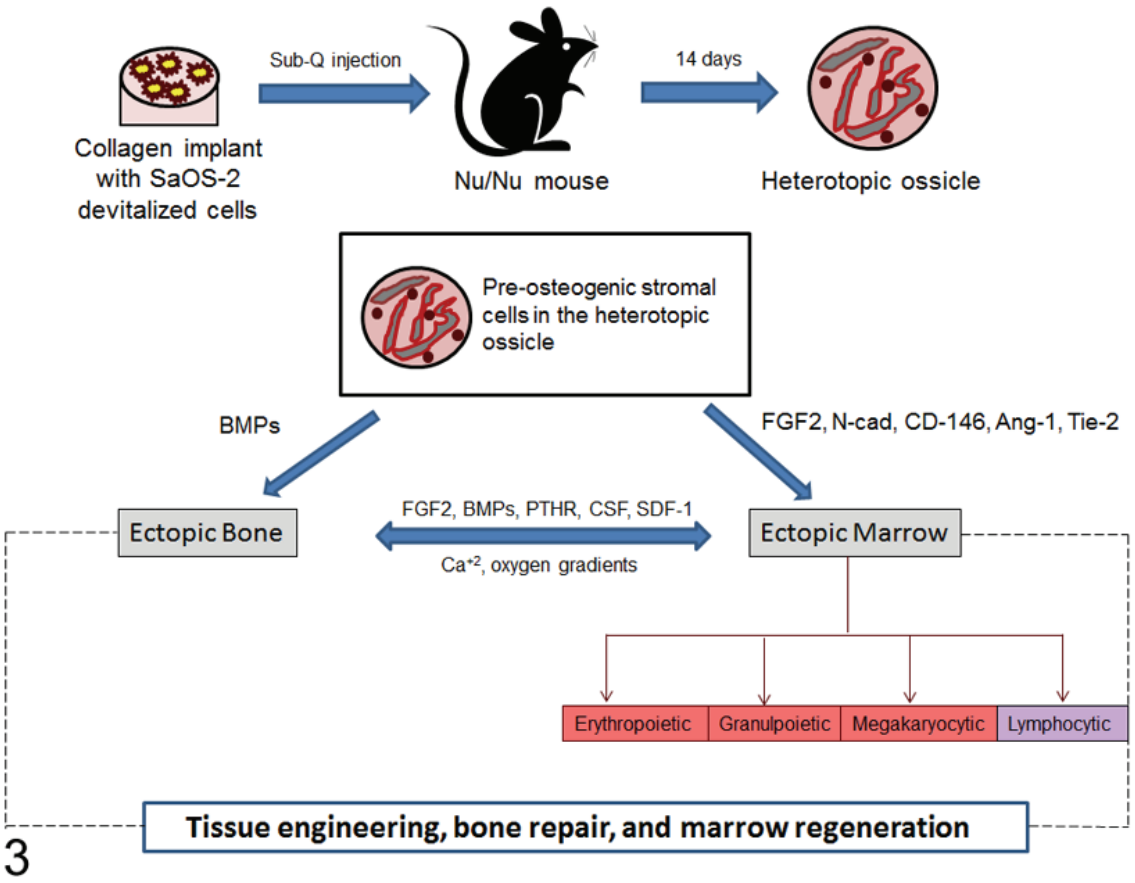

Figure 3. Schematic illustration showing induction of ectopic bone and marrow by devitalized SaOS-2 cells. We propose that collagen matrix/scaffold containing devitalized SaOS-2 cells when implanted subcutaneously into Nu/Nu mice generate a heterotopic ossicle which is able to induce *de novo* ectopic bone and marrow. SaOS-2 derived marrow contains all the major hematopoietic cell types.

be from the host's orthotopic marrow via the blood stream [14]. The maintenance of the newly formed marrow is mediated by mesenchymal stem cells of the host's subcutaneous tissue that are induced to undergo differentiation to form marrow microenvironment supportive cells, and regulate mobilization, and multilineage differentiation of HSCs [15-17]. A study by Sccahetti et al., reported that angiopoietin-1 expressing CD 146+ bone marrow stromal cells undergo osteoblastic differentiation when exposed to fibroblast growth factor (FGF2), *in vitro*, and regulate hematopoiesis via vascular remodeling, in vivo. Those authors suggested a role for CD146+ osteoprogenitor cells in bone and marrow induction [17]. Another study by Itkin et al., reported that FGF2 expands Nestin positive perivascular stromal cells, and increases expression of stem cell factor (SCF), which in turn expands hematopoietic stem cells via c-Kit

activation [18]. The differentiation status of osteoblasts is important for regulating hematopoiesis as immature osteoblasts, which have reduced expression of SDF and Ang-1 lead to bone marrow aplasia [19].

Conclusion

In conclusion, Saos-2 cells (freeze-dried and thus devitalized), or Saos-2 cell extracts, implanted subcutaneously in Nu/Nu mice, can induce normal marrow as visualized microscopically, that is 1) host-derived, and 2) contains all major hematopoietic cell types, i.e. erythropoietic, granulopoietic, megakaryocytic and lymphopoietic cells (**Figure 3**). Further study of Saos-2-induced, *de novo* hematopoiesis is needed for several reasons including potential application of Saos-2 cell extracts (a) to restore marrow hematopoiesis in clinical situations in which hematopoiesis is deficient, and (b) to promote bone repair.

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