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 immunopositive staining for 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 week 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 osseous defects in rats [8].
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
mice. Ectopic bone and normal tibias were fixed for 24 h in 4% paraformaldehyde and decalci-
fied in 10% ethylene diamine tetra-acetic acid
(EDTA) for up to 10 days and embedded in par-
affin 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 mono-
 clonal anti-human CD79a antibody (Vantana,

![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).](image)
Ectopic marrow induced by devitalized SaOS-2 cells

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, 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, lymphopoietic 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 1. Total Cell counts in marrow of ectopic bone

<table>
<thead>
<tr>
<th>Post-implantation age of Saos-2 induced ossicles</th>
<th>Total cell count/ high power field (HPF)</th>
</tr>
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<tbody>
<tr>
<td>2 weeks</td>
<td>471</td>
</tr>
<tr>
<td>3 weeks</td>
<td>540</td>
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</tbody>
</table>

Ectopic marrow induced by devitalized SaOS-2 cells

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

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