Bone Formation by Hemopoietic Tissue:
Separation of Preosteoblast From
Hemopoietic Stem Cell Function in the Rat

By Sheldon Amsel and Elisabeth S. Dell

The bone marrow preosteoblast was studied using bone formation by subcutaneously placed syngeneic grafts of hemopoietic tissue as the test for the presence of the preosteoblast. The hind limbs of rats were irradiated with 0, 300, 600, or 900 rads, and the femoral and tibial marrow were removed and grafted 4 hr–90 days following irradiation. Grafts of the irradiated marrow, adjusted to match the unirradiated grafts in number of cells or weight of tissue, showed a marked decrease to absence of bone-forming ability over the 90-day period. The hemopoietic cells of this marrow 90 days following irradiation, except for a decrease in cellularity by one quarter, were thought to be normal as judged by differential counts and the presence of hemopoietic stem cells. The interpretations of this separation of the hemopoietic from the bone-forming function of marrow are that the preosteoblast is a nonmigrating cell and that it is not derived from the hemopoietic stem cell.

In 1851 Bruns reported the presence of bone at the site of a heterotopic autograft of bone marrow. Since then this easily performed experiment has been studied by many investigators. The intriguing aspect of this type of graft is the realization that hemopoietic tissue is organogenetic. When placed subcutaneously the implanted mass of tissue undergoes a predictable sequence of cellular changes leading to the formation of a tiny bone marrow organ composed of an outer shell of lamellar bone surrounding sinusoidal hemopoietic tissue.

Since Bruns’ original description there was continual debate over whether the osteoblasts that form the bone of the graft were derived from cells in the donor hemopoietic tissue or from cells of the host. However, in 1968 Friedenstein et al. demonstrated that the progenitors of the osteoblasts (preosteoblasts) came from the donor hemopoietic tissue. Thus along with the endosteal and periosteal osteocyte and the osteoblast adjacent to the epiphysis in growing bones, marrow itself contains a cell with the potential to form bone.

We have been concerned with defining the characteristics of the preosteoblast from the hemopoietic tissue.
blast and the relationship between the bone-forming function and the hemo-
poietic function of bone marrow. The latter question resolves into whether the
preosteoblast is physiologically related to the hemopoietic stem cell. Previous
work has shown that if a rat is given a lethal, whole body dose of X-irradia-
tion (900 R), subsequent syngeneic transplants of its hemopoietic tissue up to
a period of 10 days are incapable of forming bone. Thus, an in vivo dose of
900 R stops the preosteoblast from proliferating. In the present report, only
the hind limbs were irradiated with 900 rads. This provided an experimental
animal that survived for a long period and whose femoral and tibial marrow,
90 days postirradiation, appeared morphologically normal except for a slight
reduction in cellularity. The viability of the preosteoblast in this marrow was
tested by its ability to form bone when heterotopically grafted.

MATERIALS AND METHODS

Terminology, Animals, and Irradiation

The term preosteoblast is used to indicate a precursor cell or line of cells residing in
hemopoietic tissue that has the potential to differentiate into an osteoblast and form bone.
The words transplant or graft connote the syngeneic grafting of hemopoietic tissue en bloc
to a subcutaneous site. Inbred, male, Lewis rats (Simonsen Laboratories, Gilroy, Calif.)
weighing 200 g were used in all experiments. Irradiation was performed with a 250 kV,
GE Maxi-mar III X-ray generator utilizing 0.5 mm Cu and 1 mm Al filters. The calculated
absorbed dose rate was 50 rads/min. To deliver localized irradiation, the hind limbs of
four anesthetized (i.p. Nembutal, 30 mg/kg body weight) prone rats were irradiated at a
target to dorsal skin distance of 53 cm, while the upper one-half of their bodies was
shielded with lead. For whole body irradiation a dose of 850 rads was used omitting the
shield. Following whole body irradiation rats were given ampicillin, 100 mg/kg, sub-
cutaneously daily for 7 days.

Estimation of Cellularity of Subcutaneous Graft

The technique of removing hemopoietic tissue en bloc by insertion of a polyethylene
catheter (PE 160, Clay Adams) into the shafts of the rat tibia and femur was previously
reported. The catheters, withdrawn within 3 min of killing the animal, contained a column
of hemopoietic tissue and were subjected to either of two procedures. In the first, they were
held against a 10 cm ruler and cut so that columns of hemopoietic tissue measuring a
desired length were obtained. (e.g., a 5.5 cm column contained approximately 40 mg of
tissue) The catheters (usually two pieces) were weighed, and the marrow then extruded
subcutaneously during the grafting procedure. They were then reweighed thus giving the
amount of hemopoietic tissue grafted. The second procedure determined the cellularity
of the marrow in cells per mg of tissue. A catheter containing a column of hemopoietic tissue 1–2 cm in length was weighed, and using a needle and syringe the tissue was then
air blown into 1 cc of saline. The catheter was then reweighed, giving the mg of hemo-
poietic tissue in the saline. The cells were dispersed with a Pasteur pipette, and a nucleated
cell count performed by the routine WBC method. The cells per mg of hemopoietic tissue
were calculated, and using the mg of tissue grafted an estimate of the number of cells
in the subcutaneous graft was obtained.

Test for Presence of Preosteoblast in Marrow Previously Irradiated

The hind limbs of rats were irradiated with 300, 600, or 900 rads. A control group
remained unirradiated. After 90 days, rats in each group were killed two at a time, and
the marrow from their tibias and femurs quickly removed in catheters. The column of
hemopoietic tissue in the catheters was measured, cut, and weighed as described above.
The marrow was then grafted into subcutaneous pockets of inbred rats by blowing the
tissue from the catheters using a needle and syringe. Usually the marrow from two catheters was deposited in one pocket to deliver the desired number of cells. When the marrow cellularity was reduced a longer column of tissue was used. The empty catheters were then reweighed. Two weeks later the graft site was explored, and if a macroscopic bone spicule was found the graft was considered positive and evidence for the presence of viable preosteoblasts.

Each animal killed had a small portion of its hemopoietic tissue dispersed to measure its cellularity quantitatively as described above. Three tibias and three femurs from each of the four groups were fixed in 10% formalin, and hematoxylin and eosin sections were obtained after decalcification. Multiple marrow smears from each group were stained with Wright's stain.

In an additional experiment the hind limbs of animals were irradiated with 900 rads. Groups of animals were killed at 4 hours, 1, 5, 11, 30, 60, and 90 days. The marrow was removed in catheters, weighed, syngeneically transplanted and the catheters reweighed as described above. Marrow smears were made. Approximately 50 mg of marrow were deposited subcutaneously at each graft site. Marrow cellularity was not quantitatively determined except in the 90-day group. Again the graft site was explored after 2 wk, and bone formation recorded.

Test for Presence of Hemopoietic Stem Cell (HSC) in Marrow Previously Irradiated

Ten rats that 90 days previously had been given 900 rads to their hind limbs were killed two at a time, and their tibial and femoral marrow removed in catheters. The marrow was then blow into 10 cc of saline, and the cells dispersed and counted as described above. Viability by the trypan blue dye exclusion test was 95–98%. After a cell count, $50 \times 10^6$ nucleated marrow cells were injected into the tail vein of each recipient rat. The recipient rats were inbred and had been exposed to a whole body dose of 850 rads within 6 hr of the injection. One control group received 850 rads and no cells. A second control group received 850 rads and $50 \times 10^6$ nucleated cells from a nonirradiated inbred rat. Rats surviving greater than 30 days were considered to have received HSC from the injected cell suspension. Any rat dying within 7 days of whole body irradiation was excluded from the assay, since a gastrointestinal death could not be excluded.

RESULTS

Study of Cellularity and Bone-Forming Ability of Rat Marrow After Localized Irradiation

The results are summarized in Table 1 and Fig. 1. Ninety days following irradiation with 300, 600, or 900 rads the cells per mg of hemopoietic tissue in the lower extremities were significantly reduced when compared with the unirradiated rats ($p < 0.001$). This 20%–30% decrease in cellularity was corroborated by the slightly reduced cellularity of the femurs and tibias on hematoxylin and eosin sections. The differential counts of 500 cells from the irradiated and control rats were similar (Table 1), and all cell lines appeared to differentiate normally. Similar numbers of marrow cells from those four groups weregrafted subcutaneously into syngeneic rats. The bone spicules formed by the control marrow were rigid, irregularly shaped rods measuring 1–11 mm in length and 1–2 mm in diameter. Spicules formed by the irradiated marrow were 1–2 mm in length and 0.5 mm in diameter. The experiment demonstrates that grafts of marrow tissue taken from rat long bones 90 days after irradiation with 300, 600, or 900 rads and containing hemopoietic cells matched in number and kind (Tables 1 and 3) with unirradiated marrow grafts have a reduced capacity to form bone.
Grafts of approximately 50 mg of hemopoietic tissue removed 4 hr–90 days following irradiation of the hind limbs with 900 rads also had a reduced capacity to form bone (Table 2). Wright’s stained smears of this hemopoietic tissue showed marked hypocellularity at 1 and 5 days, moderate hypocellularity at 11 days, and normocellularity at 4 hr and 30 days. This pattern of cellular changes following irradiation has been previously reported. The return of marrow cellularity evident at 10 days and complete at 30 days is most likely caused by migration of the HSC into the injured marrow and its subsequent proliferation. Endogenous recolonization cannot be excluded from also occurring. The reason for the positive grafts within the first 11 days is unclear. However over the 90-day period only seven of 79 grafts formed bone, and no bone formation occurred past day 30. It seems reasonable to conclude that if the preosteoblast had the capacity to migrate and proliferate like the HSC, the ability of the marrow to form bone would have returned to a greater degree within 90 days.

Table 1. Study of Cellularity and Bone-Forming Ability of Rat Marrow 90 Days After In Vivo Localized Irradiation

<table>
<thead>
<tr>
<th>Dose (rads)</th>
<th>0</th>
<th>300</th>
<th>600</th>
<th>900</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellularity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nucleated cells × 10⁶/mg tissue (mean ± SE)</td>
<td>1.6 ± 0.04</td>
<td>1.1 ± 0.04</td>
<td>1.2 ± 0.06</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>Number of counts performed</td>
<td>55</td>
<td>18</td>
<td>17</td>
<td>30</td>
</tr>
<tr>
<td>Number of rats counted</td>
<td>18</td>
<td>18</td>
<td>13</td>
<td>16</td>
</tr>
<tr>
<td>Bone-forming ability</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg of tissue in graft (mean)</td>
<td>30</td>
<td>40</td>
<td>41</td>
<td>42</td>
</tr>
<tr>
<td>Number of cells in graft (× 10⁴)</td>
<td>48</td>
<td>44</td>
<td>49</td>
<td>54</td>
</tr>
<tr>
<td>Number of grafts performed</td>
<td>31</td>
<td>22</td>
<td>19</td>
<td>18</td>
</tr>
<tr>
<td>Positive grafts</td>
<td>27(87%)</td>
<td>6(27%)</td>
<td>3(16%)</td>
<td>0</td>
</tr>
</tbody>
</table>

*Myeloid to erythroid to lymphoid ratio of 500 cells given as percentages.
Table 2. Bone Formation by Heterotopic of Rat Marrow Following In Vivo Localized Irradiation With 900 Rads

<table>
<thead>
<tr>
<th>Time of Graft Following Irradiation</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 hr</td>
</tr>
<tr>
<td>Mg of tissue grafted (mean)</td>
<td>48</td>
</tr>
<tr>
<td>Number of grafts</td>
<td>10</td>
</tr>
<tr>
<td>Positive grafts</td>
<td>2 (20%)</td>
</tr>
</tbody>
</table>

Test for Presence of HSC in Rat Marrow 90 Days Following Localized Irradiation

The results are summarized in Table 3. The 30-day survival conferred upon lethally irradiated rats by \( \text{50} \times 10^6 \) normal syngeneic marrow cells was roughly the same as the survival produced by \( \text{50} \times 10^6 \) syngeneic cells from marrow irradiated 90 days before with 900 rads. The results suggest that cell suspensions of unirradiated and irradiated marrow, when adjusted to contain equal numbers of cells, contain similar numbers of HSC.

DISCUSSION

The experiments indicate that 3 mo following localized marrow exposure to 300, 600, or 900 rads there was a reduction in marrow cellularity by approximately one quarter. Wright’s stained smears of this same marrow demonstrated normal appearing cells with the same percentage of myeloid, erythroid, and lymphoid cells as in a normal marrow. The presence of hemopoietic stem cells also substantiates the marrow being hemopoietically normal in a qualitative sense. However, when this same irradiated hemopoietic tissue was grafted subcutaneously, with the cellularity (Table 1) and weight (Table 2) of the graft adjusted to match the control unirradiated grafts, there was a marked decrease in bone-forming ability. This split in bone-forming ability and hemopoiesis is especially striking in the marrow 90 days following irradiation with 900 rads, where there was only a 20% loss in cellularity, retention of HSC activity, microscopically normally apering hemopoietic cells but complete loss of bone-forming function. This is interpreted as follows. The preosteoblasts and HSC, after exposure to an in vivo dose of 900 rads, lose their ability to proliferate. Additional HSC then migrate from unirradiated marrow.

Table 3. 30-Day Survival of Lethally Irradiated Rats Injected With Syngeneic Hemopoietic Cells Collected From Long Bones 3 Mo After Localized Irradiation With 900 Rads

<table>
<thead>
<tr>
<th>Whole Body Irradiation (rads)</th>
<th>No. of Nucleated Marrow Cells Injected</th>
<th>30-Day Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test rats</td>
<td>850</td>
<td>( \text{50} \times 10^6 )</td>
</tr>
<tr>
<td>Control rats</td>
<td>850</td>
<td>( \text{*50} \times 10^6 )</td>
</tr>
<tr>
<td>Control rats</td>
<td>850</td>
<td>None</td>
</tr>
</tbody>
</table>

*Normal marrow cells from unirradiated inbred rats.
to the irradiated aplastic marrow, proliferate, and eventually restore cellularity to levels approaching normal. The preosteoblasts, unable to proliferate or migrate (see Results), have now been functionally eliminated from this haemoipoietic tissue. When the tissue is heterotopically grafted, a stimulus that usually causes intense preosteoblast proliferation, no osteoblasts can be formed, and as a result no bone is produced.

Since the HSC seems to be functioning adequately in the absence of preosteoblast function, a corollary of the above interpretation is that the preosteoblast is not derived from the HSC. The experiments, however, do not exclude the reverse, i.e., a common cell precursor or the preosteoblast at an unirradiated site giving rise to the HSC. There is some indirect experimental evidence for the latter, for following mechanical depletion of marrow from a long bone or subcutaneous grafting of marrow, both of which are associated with local preosteoblast proliferation, the repopulating haemoipoietic tissue can be derived from local cells.

The nature of the cell line from which the preosteoblast is derived and the mode of its differentiation into an osteoblast has never been satisfactorily answered. The various possibilities have been discussed by Friedenstein et al. Urist et al. have demonstrated that mesenchymal cell populations of certain tissues (muscle, subcutaneous tissue, kidney calyx, etc.) can be induced to differentiate into osteoblasts and form bone. The inducing agent used was decalcified lyophilized bone; however, others have used urinary bladder epithelium. The inducing agent is thought to release an acid stable principle that causes the mesenchymal cells to differentiate into osteoblasts. The counterpart of the mesenchymal cell in bone marrow is probably the preosteoblast. The marrow preosteoblast differs however in that it can form bone when placed in any heterotopic location. Marrow therefore contains the inducing agent and the osteogenic competent cell within its own tissue. The mechanical trauma of the heterotopic graft or the in situ marrow disruption by a trocar probably triggers its release. The nature of this inducing agent in marrow, its method of action, and the properties of the nonmigrating cell on which it acts remain unexplained.

REFERENCES

9. Amsel, S., and Dell, E. A.: Response of the preosteoblast and stem cell of rat bone


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