Non-adherent Bone Marrow Cells Are a Rich Source of Cells Forming Bone *in Vivo*

(bone formation in kidneys / bone marrow / non-adherent bone marrow / stromal cell cultures / histocompatibility barrier)

K. H. WŁODARSKI, R. GALUS, P. WŁODARSKI

Chair and Department of Histology and Embryology, Center of Biostructure, Medical Academy at Warsaw, Poland

Abstract. Syngeneic, allogeneic and xenogeneic (rat) freshly isolated bone marow cells + stromal cell cultures maintained *in vitro* for 10–30 days, as well as non-adherent cells removed from these cultures on 3rd–4th day were injected into the kidney parenchyma of mice, immunosuppressed with hydrocortisone. In syngeneic grafts the immunosuppression was omitted. In all transplant systems bone tissue was formed inside the kidney with 20% to 32% variation. Bone produced by allogeneic and xenogeneic cells is subject to rejection when immunosuppression ceases, as the bone formed is of donor origin. The „floating" cells, regardless of the transplant system, normally discarded during media replacement, turned out to be efficient bone producers. This notion is of practical implication when bone marrow cells are used for bone healing.

Mesenchymal stem cells (MSC) proliferate and differentiate into connective tissues, including bone marrow stroma, adipocytes, bone and cartilage. They reside amongst others in the bone marrow stroma.

During *in vitro* culture they proliferate to confluence in 2–3 weeks and can be passaged for several generations, maintaining a non-haematopoietic phenotype. To estimate the frequency of MSC, the colony-forming unit fibroblast assay (Friedenstein, 1976) is widely used. The estimated frequency of MSC in human bone marrow is determined as ca 0.01% of nucleated cells (Jones et al., 2002). Murine bone marrow expresses a similar frequency ratio (Meirelles and Nardi, 2003). Bone marrow MSC provide a microenvironment for haemopoietic cell differentiation, but are distinct from the haemopoietic fraction.

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Corresponding author: Krzysztof H. Włodarski, Chair and Department of Histology, Center of Biostructure, Medical Academy, 02-004 Warszawa, Chałubińskiego 5, Poland. Tel.: (+48) 226281041 (ext. 21 or 47); Fax: (+48) 226295282; e-mail: kwlodar@ib.amwaw.edu.pl

Abbreviations: MEM – minimal essential medium; MSC – mesenchymal stem cells.

On transplantation into subcutaneous or intramuscular sites, cultured adherent bone marrow cells and stroma-derived cell lines produce bone (Benayahu et al., 1992; Haynesworth et al., 1992). Osteoprogenitor cells comprise approximately 1% of plated adherent cells (Aubin, 1999). When cultured in the presence of beta-glycerophosphate and vitamin C, they form a mineralized extracellular matrix, which can be demonstrated using Alizarin S stain or Van Kossa techniques (Maniatopoluos et al., 1988; van Vlasselaer et al., 1993; Ohgushi et al., 1996; Schecroun and Delloye, 2003). In the presence of dexamethasone they form foci of adipocytes. A switch in stromal cell differentiation from the osteoblastic to the adipocytic pathway is postulated in osteoporosis (Verma et al., 2002). Also, in the rat, the ability of marrow stromal cells for osteogenesis decreases, but the ability of adipogenesis increases with age (Chen et al., 2003). The reciprocal relationship between osteoblastogenesis and adipogenesis can be manipulated *in vivo* in order to improve bone formation (Ahdjoudj et al., 2001).

In rat marrow stroma, there are at least two classes of osteoprogenitor cells: those differentiating in the absence of added dexamethasone and those requiring glucocorticoid to differentiate (Aubin, 1999). There are reports, however, that rat fibroblast-like stromal cells lack osteoblast markers (Simmons et al., 1991).

Human MSC are composed of a heterogenous mixture of cells at varying stages of differentiation and with distinct osteogenic potential (Phinney et al., 1999).

The attempts to use autologous bone marrow cells or cultured marrow stromal cells to correct bone defects gave promising results in experimental animals and humans (Niedźwiedzki et al., 1993; Lazarus et al., 1995; Krzymański et al., 1997; Bruder et al., 1998; Horwitz et al., 1999; Krebsbach et al., 1999; Oreffo and Triffitt, 1999).

Heterotopic osteogenesis by bone marrow and marrow stroma has been reported by numerous authors. The osteogenic potential of cells can be assessed prior to transplantation in many ways, e.g. by testing for the expression of bone markers, the response to the osteotropic agents, or by their ability to induce mineralized

bone nodules. In our laboratory we verify the osteogenic properties of cells by *in vivo* assesment after grafting them into mouse kidney parenchyma. Historically, it was established that the kidneys lack inducible osteogenic precursor cells (Włodarski, 1978); thus bone formed within the kidney has to originate from the grafted cells and could be subject to tissue rejection.

To assess the efficacy of this assay in measuring the bone inducing potential of cultured bone marrow stromal cells prior to their being grafted into mouse kidney or human bone defect sites, fresh syngeneic, allogeneic and xenogeneic bone marrow cells, plus their cultured stromal cells, were grafted into the kidney cortex or under the kidney capsule of immunosuppressed mice. The presence of induced bone was examined histologically at 8–10 days post transplantation.

We have observed that non-adherent cells, removed from primary bone marrow culture, after 3–4 days maintain the ability to grow as stromal cells (in human marrow culture such „floaters" have produced only one third of the colonies compared with primary cultures – Włodarski, unpublished). To examine whether amongst the „floaters" are cells capable of generating bone *in vivo*, „non-adherent cells", after 2–3 days in culture, were grafted into kidneys to test their osteogenic potential.

Material and Methods

Animals and treatments

BALB/c, CFW/Ll inbred and MIZ outbred strains of 2–4 months old mice weighing 18–25 g, and Wistar or Lewis inbred strains of rats aged 2–6 months were used. Institutional guidelines for the care and treatement of laboratory animals were observed. The mice were kept up to 5 per cage with free access to mouse chow and water ad libitum. Mice were anaesthetized with ether, sacrificed by cervical dislocation. Rats were sacrificed by an ether overdose.

Collection of bone marrow cells

Bone marrow cells were flushed out from femoral cavities by syringing with minimal essential medium (MEM) (Gibco, Paisley, UK). Bone marrow from several animals was centrifuged, washed with MEM tissue culture medium and the cell suspension used either for transplantation into the kidney or for stromal cell culture.

Usually, the contents of two murine femoral cavities were suspended in the MEM medium and injected in 0.1–0.15 ml into two surgically exposed kidneys. A similar procedure was applied to rat bone marrow.

Tissue culture and cell transplantation

The bone marrow cell suspension harvested from mice or rats was cultured in MEM medium plus 10% foetal calf serum and antibiotics (Gibco) using Falcon plastic flasks. Two–four days later the medium was replenished. The non-adherent cells were collected, centrifuged, and the cell suspension containing 3–4 x 10^6 of nucleated cells was injected into the kidney parenchyma or under the renal capsule.

Marrow stromal cells were cultured from 10 to 30 days. When confluent, they were trypsinized and detached with a rubber scraper, washed twice in phosphate-buffered saline and 0.7–1.1 x 10^6 of nucleated cells were injected into mouse kidneys. The viability of cells was ca 80% as estimated by the trypan blue exclusion test.

Treatment of animals

The animals were anaesthetized with ether, wounds closed with 2–0 Dexon „S" polyglycolic acid surgical suture thread. Recipients of allogeneic and xenogeneic grafts were given, subcutaneously, 3.75–5.0 mg of hydrocortisone acetate (Jelfa, Jelenia Góra, Poland), immediately after surgery. The immunosuppressive effect was monitored by spleen weight, determined on the sacrifice. In syngeneic cell grafts, no immunosuppression was used. Non-fractioned bone marrow cell harvest from one mice (ca 15 x 10^6 of nucleated cells) was usually grafted into 2–3 recipients.

Histology

Animals were sacrificed by cervical dislocation 8–10 days post graft, the kidneys fixed in Bouin solution, decalcified in EDTA, embedded in paraffin wax, and 10 μm serial sections were stained with haematoxylin and eosin and examined for the presence of grafted cells and bone. No Van Kossa reaction was applied, as the tissues were decalcified before histological procedure. In some cases, when bone trabeculae were resorbed due to the histoincompatibility reaction, the PAS (periodic acid-Schiff) reaction was performed to visualize the bone matrix. The efficiency of the graft cell response was determined by the presence of induced bone tissue in the kidney.

The numbers of grafts performed and the number of grafts producing bone within kidneys are presented in Table 1. Altogether 1373 grafts were examined, including kidneys exhibiting macroscopic abscesses or necrosis.

Results

Syngeneic bone marrow transplantation

1. Freshly isolated bone marrow cell grafts from 223 BALB/c or CFW/Ll mice into syngenic recipients of the same sex gave positive results in fifty cases (50/223; 22%). Bone trabeculae were covered with osteoblasts and no osteoclasts were seen (Fig. 1). In a proportion of cases, the grafted bone marrow cells, identified by the presence of megakaryocytes, were found beneath kidney capsules, with no bone seen.

2. Non-adherent bone marrow cells, after 48–72 h in culture, were collected from 2–3 days old primary

Table 1. The incidence of bone formation in mouse kidneys following transplantation of syngeneic, allogeneic and xenogeneic rat bone marrow cells expressed as a percentage of kidneys with bone tissue versus the number of grafts performed. The kidneys were examined histologically for the presence of bone tissue 8–10 days after cell grafting. In parentheses, nominator indicates the number of grafts producing bone tissue in the kidney, denominator – the number of grafts performed.

Type of graft	Fresh isolated bone marrow cells Whole bone marrow cell suspension	Non-adherent bone marrow cells after 48–72 h in culture	Bone marrow stromal cells grown *in vitro* for 10–30 days
Syngeneic	22% (50/223)	33% (59/176)	20% (44/211)
Allogeneic*	15% (27/181)	21% (13/ 63)	18% (7/38)
Xenogeneic (rat) *	22% (45/205)	32% (33/104)	16% (27/172)

*) recipient mice were immunosuppresed with a single dose of 3.75–5.0 mg of hydrocortisone.

marrow cultures and grafted into 176 syngenic hosts. They produced bone in 59 cases (59/176; 33%) (Fig. 2). Also in this group, megakaryocytes were seen occasionally.

3. *Stromal cell cultures.* The stromal cells cultured *in vitro* were a heterogenous population, composed of fibroblast-like cells, cobblestone-like cells, macrophages and sometimes adipocytes. Adipocytes appeared from day 12 onwards. These cultures were relatively resistant to trypsin digestion, so they were recovered by scraping.

Only in 44 cases out of the 211 grafts performed, bone trabeculae were found (44/211; 20%). Bone trabeculae were covered with osteoblasts, and no megakaryocytes were seen (Figs. 3 and 4).

Allogeneic bone marrow cell transplantation

1. Freshly isolated whole bone marrow cells. Bone marrow cells were grafted between outbred MIZ strains of mice and in the combination CFW/Ll and BALB/c and *vice versa*. The results are presented collectively.

In 27 out of 181 grafts performed, bone trabeculae were found (27/181; 15%). Bone trabeculae were infiltrated with mononuclear cells, and many osteocytic lacunae were empty, indicating the rejection of allogeneic bone.

2. *Non-adherent bone marrow cells after 48–72 h in culture* were grafted into immuosuppressed mice producing bone in 13 out of 63 grafts performed (13/63; 21%). The morphology of bone was similar to that of fresh bone marrow cell grafts. Kidneys were infiltrated with mononuclear cells, accumulated around arterial vessels.

3. *Stromal cell cultures.* The incidence of bone formation following adherent cells grown *in vitro* for 10–30 days was 18% (7/38) (Fig. 5). Bone trabeculae were infiltrated with mononuclear cells of moderate intensity, and no osteoclastic resorption has been observed.

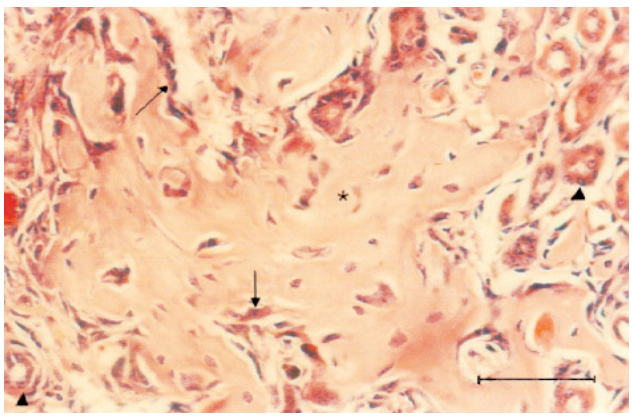

Fig. 1. Bone trabecula (*) within kidney medulla formed by whole syngeneic bone marrow, 12 days post cell inoculation. Arrows point to the osteoblasts. Arrowheads point to the kidney tubules. Haematoxylin-eosin staining, scale bar 100 μm

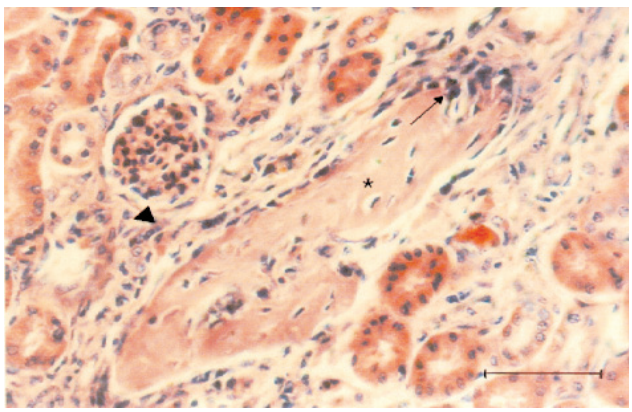

Fig. 2. Bone trabecula (*) in kidney cortex following administration of non-adherent syngeneic bone marrow cells after 48 h in culture, 10 days post grafting of floating cells. The edge of trabeculae is covered by osteoblasts (arrow). Arrowhead points to the kidney glomerulus. Haematoxylin-eosin staining, scale bar 100 μm

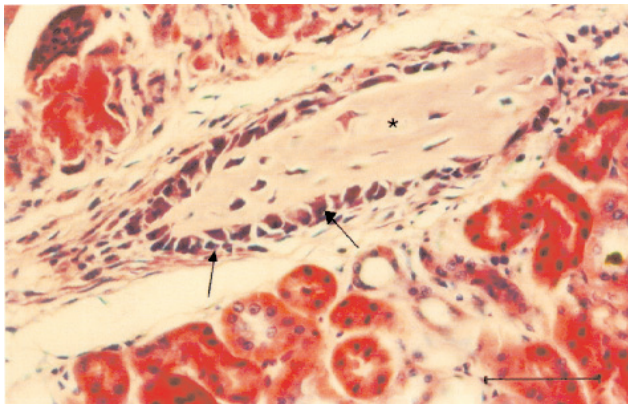

Fig. 3. Bone trabecula (*) in the kidney formed following syngeneic bone marrow stromal cell culture *in vitro* for 12 days, 9 days post cell engraftment. Bone trabecula is covered by the rim of osteoblasts (arrows). Haematoxylin-eosin staining, scale bar 100 µm

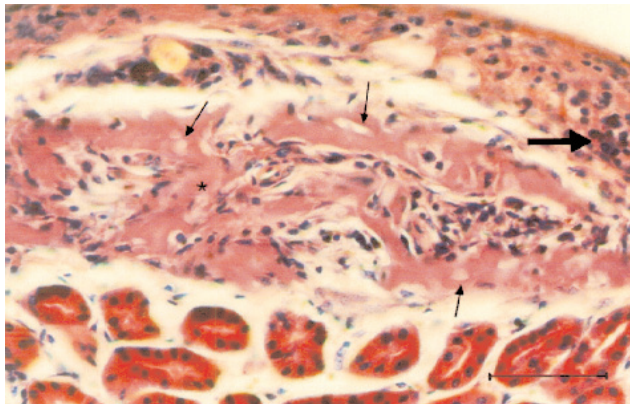

Fig. 6. Bone trabecula (*) formed by xenogeneic (rat) freshly isolated bone marrow, 9 days post cell grafting. Some osteocytic lacunae are empty (arrows) and mononuclear cells are infiltrating xenogeneic bone (bold arrow). Haematoxylin-eosin staining, scale bar 100 µm

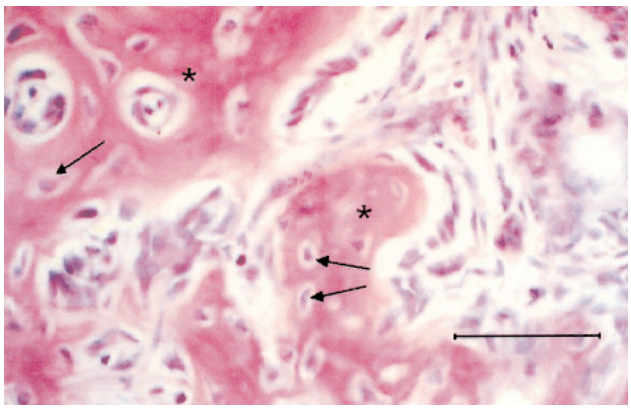

Fig. 4. Bone trabecula (*) formed by syngeneic stromal bone marrow cells, cultured *in vitro* for 11 days; 9 days post cell grafting into the kidney parenchyma. Arrows point to the osteocytic lacunae filled with osteocytes. Strong PAS reaction in the bone matrix. PAS reaction and haematoxylin staining, scale bar 100 µm

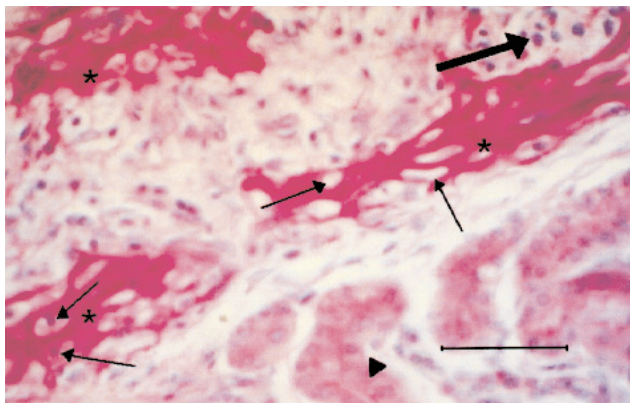

Fig. 7. Bone tissue (*) formed in the kidney parenchyma following whole xenogeneic (rat) bone marrow cell suspension. Bone is resorbed by host mononuclear cells (bold arrow) and many osteocytic lacunae are empty or are containing dying osteocytes (arrows). Arrowheads point to the kidney tubules. PAS reaction is strongly positive in the bone matrix. Counterstained with haematoxylin. Scale bar 100 µm

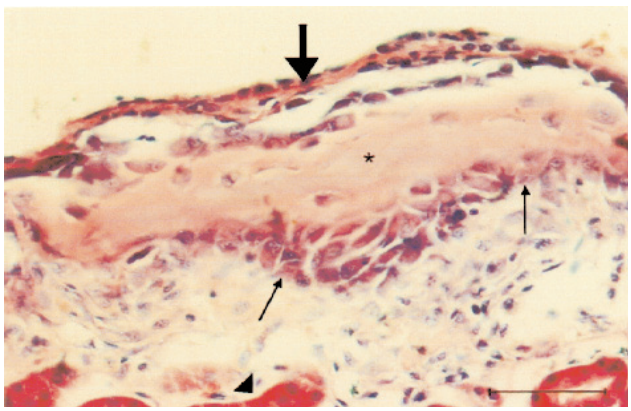

Fig. 5. Bone trabecula (*) under the renal capsule (bold arrow) formed by allogeneic bone marrow stromal cell cultures, no sign of bone rejection. Eleven days post cell grafting. Osteoblasts are indicated by arrows. Kidney tubules are marked by arrowheads. Haematoxylin-eosin staining, scale bar 100 µm

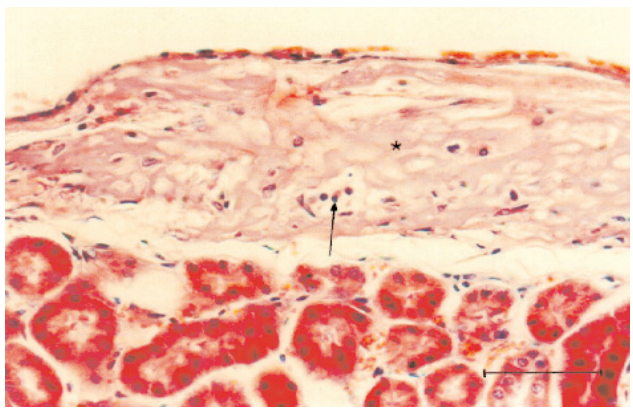

Fig. 8. Bone tissue (*) under a renal capsule formed by non-adherent (floating) xenogeneic bone marrow cells after 48 h in culture, 9 days post cell grafting. Most osteocytes died leaving osteocytic lacunae empty. No osteoclasts are seen and bone matrix is slightly infiltrated by mononuclear cells (arrow). Haematoxylin-eosin staining, scale bar 100 µm

Xenogeneic (rat) bone marrow cell transplantation

1. *Freshly isolated bone marrow cells.* The incidence of bone formation following Wistar and Lewis rat bone marrow cell transplantation into hydrocortisone-treated mice was ca 22% (45/205). Bone trabeculae were extensively damaged by infiltrating mononuclear cells, heavy infiltrations were observed around kidney arteries. No osteoclasts were seen (Figs. 6 and 7).

2. Bone marrow cells non-adhering after 48–72 h of cultivation. The incidence of bone formation by this group was 32% (33/104), and bone morphology did not differ from that of fresh xenogeneic marrow cells (Fig. 8).

3. *Rat stromal cell cultures. In vitro* grown rat stromal cell cultures displayed a high degree of polymorphism, with fibroblast-like cells dominating. As with murine stromal cells, they were relatively resistant to trypsin digestion and were recovered from flasks by scraping. On transplantation into kidneys, they produced bone in ca 16% of cases (27/172) and the xenogeneic bone was strongly infiltrated and resorbed by mononuclear cells.

Immunosuppressive effect of hydrocortisone

To assess the immunosuppressive effectiveness of a single dose of 3.75–5.0 mg of hydrocortisone (doses were adjusted to the weight of animals), the spleen weight was estimated at autopsy and mean weight ± SD was calculated for experimental groups. The mean weight of spleen of inbred CFW/Ll and BALB/c mice of both sexes, injected with syngeneic bone marrow cells, but not receiving hydrocortisone, was 137 mg ± 34 mg (N = 92). The spleen weight of recipients of allogeneic cells following hydrocortisone treatment was 88 mg ± 52 mg (N = 22), and that of xenogeneic cells was 67 mg ± 32 mg (N = 134).

Discussion

Following transplantation of syngeneic, allogeneic and xenogeneic bone marrow cells into the kidney, parenchymal bone formation was observed in a varying proportion of cases. Both, freshly isolated and non-adherent bone marrow cells, as well as marrow stromal cells cultured *in vitro* for 10–30 days can rarely produce bone. This relatively low incidence of bone detection in the kidney could be attributed to the heterogeneity of grafted cells, their loss from the graft site (= insufficient quantity), by failing to detect minute bone trabeculae (only every 10–20th section was inspected), as well as by not excluding from the count kidneys with necrosis and infection. However, for the sake of reliability, nothing was excluded; thus the results presented give an insight into the sensitivity and limitations of such an assay. The large numbers of grafts performed justifies our conclusion that non-adherent cells contain a population able to produce bone *in vivo* also across the histoincompatibility barrier.

Bone formation in the kidney was obtained across the histoincompatibility barrier, provided an immunosuppression was applied. The efficacy of the procedure used was judged by a reduction in spleen weight (ca 50%) and by assessing the inflammatory response in the kidneys. Transplantation of allogeneic and xenogeneic marrow under the kidney capsule was reported earlier (Gurevitch et al., 1999), but the transplant was in the form of marrow plug and not of cell suspension. The authors used X-ray irradiation and cyclophosphamide as an immunosuppressor. Osteogenic expression of allogeneic rat bone marrow cells in porous hydroxyapatite ceramics was reported by Akahane et al. (1999), and Nilsson et al. (1999) found that allogeneic murine whole bone marrow contains cells that engraft and become competent osteoblasts capable of producing bone.

Also Ohgushi and Okumura (1990) have grafted rat and human marrow cells filling porous ceramics into athymic mice, producing bone formation.

As expected, better results were obtained with syngeneic bone marrow than with the allogeneic one. Surprisingly, good results were obtained with xeneogeneic bone marrow cells, almost equal to syngeneic grafts.

The stromal cells, commonly considered as a rich source of osteoblast progenitors, unexpectedly induced no more bone than whole bone marrow cell grafts.

Consistently good results in all transplant systems were obtained when the non-adherent marrow cell population was grafted (33%, 21% and 32% for syngeneic, allogeneic and xenogeneic grafts), i.e. that non-adherent cells are relatively abundant in osteoprogenitor cells and that adherent stromal cells are not the best source of osteogenic cells, as is generally thought. It is not clear why the best results were obtained with stromal cell-depleted marrow. One possible explanation is that amongst stromal cells, there are regulatory cells that inhibit osteogenesis by osteoprogenitors resident in the marrow. Such speculation is based on a lack of osteogenesis within bone marrow. Prevention of osteogenesis enables haematopoiesis to proceed. Removal of fibroblasts would prevent the smothering of bone-forming cells in the marrow. In the intact marrow, stromal cells could play a role in preventing osteogenesis in haematopoietic marrow.

Even if the difference in bone formation between freshly isolated bone marrow cells versus marrow cells depleted of their stromal cell population is insignificant, the results reported here indicate that floating cells, removed from primary cultures during media replenishment, are rich in osteoprogenitor cells.

Several authors reported earlier that the non-adherent fractions of rodent and human bone marrow contain non-haematogenous cells which express osteoblastic

markers (Falla et al., 1993; Long et al., 1994; Oyajobi et al., 1999). The non-adherent cells, grown in stirred suspension culture, contain more osteoblast progenitors than adherent cultures (Baksh et al., 2003). Floating cells from rodent marrow cultures are able to produce adherent stroma (Włodarski, unpublished results). Is is possible that adherent cells remain in the non-adherent fraction and they could contribute to its osteogenic potential. So far, no attempt has been made to prove the osteogenic capacity of these „floaters" *in vivo*.

Our intention is to report the phenomenon that non-adherent cells, cultured from rodent bone marrow, exhibit osteogenic capacity *in vivo* as demonstrated by the definitive histological findings. This observation could be of practical value when bone marrow cells are used as an aid for bone reconstruction. The non-adherent cells should be saved for this reason and future work could examine their characteristic profile.

Bone produced by allogeneic and xenogeneic bone marrow cells is subject to rejection by the host immune system. When immunosuppression ceases, such bones are invaded and resorbed by mononuclear cells, as the bone formed in this system is of donor origin. Destruction of bone is more intense for xenogeneic bones than for allogeneic bones and does not occur in syngeneic bones.

This system enables the osteogenic properties of cells to be tested, e.g. of human bone marrow cells prior to their use for bone reconstruction.

Our results are in line with Long et al. (1994) and others (Falla et al., 1993; Oyajobi et al., 1999). Their observation was that non-adherent human bone marrow is markedly enriched in cells that express osteogenic markers. We postulate that this is also true for rodent bone marrow and thus we report that such cells are able to produce bone *in vivo*.

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