Donor origin of the in vitro hematopoietic microenvironment after marrow transplantation in mice

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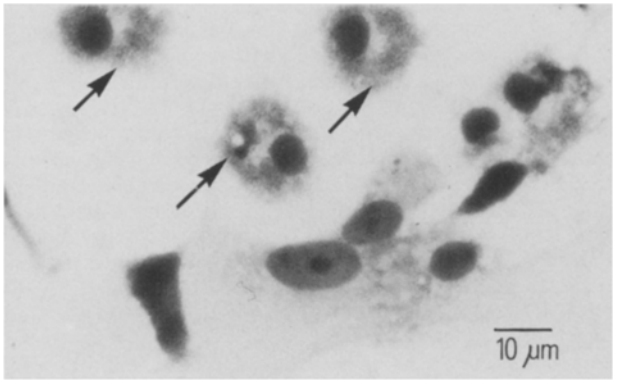
Summary: Bone marrow stroma from radiochimeric mice was established in culture. The polymorphic enzyme glucose phosphate isomerase (GPI) was used to determine the proportions of donor and recipient present in the original bone marrow and in cultured stroma. Bone marrow initially containing 95% donor GPI, when cultured and subsequently passaged for up to 8 weeks remained about 70% donor GPI. We conclude that many cultured stromal cells are donor derived in our radiochimeras and these are probably of hematopoietic origin.

The long term culture of hematopoietic bone marrow is dependent on the development of an adherent stromal cell layer[1]. At present the cells that comprise this layer are loosely defined and their inter-relationships and origins are unclear. A recent report by Keating et al.[2] suggested contrary to earlier work[3] that human bone marrow micro-environmental stroma was transplantable. To determine the origin of stromal cells in culture we examine those produced from the bone marrow of radiochimeras in the first stage of culture by the method of Dexter et al.[1].

Materials and methods. CBA/H*T6* mice and their congenic strain CBA/H*T6* Ga differ at the *Gpi-1* locus[4]. Reciprocal bone marrow transplants between these 2 strains were carried out by intravenous injection of 2×10^6 bone marrow cells into lethally x-irradiated (900 rad) recipients. These animals were killed 22 weeks later and duplicate bone marrow cultures were set up for each mouse. The method consisted of flushing the marrow from a single femur into a 25 cm^2 Falcon flask with 10 ml of Eagle's medium (Wellcome) containing 10% foetal calf serum, antibiotics, glutamine and 10^{-7}M hydrocortisone. Cultures were fed weekly by replacing half the medium. Cells were harvested by 0.05% EDTA and 0.1% trypsin. The proportions of donor and recipient GPI, a ubiquitous glycolytic enzyme, were determined by a quantitative electrophoretic method[5]. Cultured stromal cells were fractioned[6] by adding 40 mg of carbonyl iron (particle size 4.5–5.2 μM) to the culture flask and 1 hour later cells were harvested using trypsin/EDTA. Cells containing iron were recovered using a magnetic needle and these were then lysed with distilled water for GPI determination.

Results and discussion. To establish that a competent microenvironment for in vitro hematopoiesis was created by our culture method, a non-chimeric marrow was cultured and at 2 weeks after the first inoculum a second identical addition of bone marrow was made. Table 1 compares in lethally x-irradiated recipients the results of treatment by 'hematopoietic' cultured cells and primary cultured 'stromal' cells. The results confirm that spleen colony forming units are established by a second addition of bone marrow on to our stromal cultures and detection of donor GPI is a sensitive indicator of colonization.

Table 2 shows the proportion of donor GPI found in bone marrow of radiochimeric mice and also the donor GPI content of stromal cultures from these mice. A large proportion of the total GPI activity was derived from the original donor even after 8 weeks in culture and 3 passages. At this stage the cul-



Cultured bone marrow stroma showing 2 types of cell, esterase positive (non-specific E.C.No.3.1.1.1) macrophage-like cells (indicated by arrows) and an epithelioid cell which is negative for this enzyme. These cells were fixed with formol calcium in the flask then incubated with 1.25 mM 1-naphthyl acetate, 3.5 mM pararosaniline, 16 mM sodium nitrite in 60 mM phosphate buffer at pH 5.8 for 30 min at 37°C. The cells were then counterstained with 2% aqueous methyl green.

Table 1. Transfer of cells from hematopoietic and stromal cultures measured by spleen colony formation and percentage of donor GPI in whole spleen and bone marrow homogenates

Donor cells	No. of mice	CFUs	% Donor GPI Spleen	Bone marrow
Hematopoietic culture	7	11.6 (1.8)	80.4 (7.9)	57.1 (9.6)
Bone marrow stromal culture	12	0	< 5	< 5

Congenic mice differing from the donor mice at the *Gpi-1* locus were lethally x-irradiated and given 2×10^6 cells i.v. from either marrow cultured for 2 weeks after a single addition of bone marrow (stromal culture) or marrow cultured for 4 weeks with a second addition of bone marrow added at 2 weeks (hematopoietic culture). Recipient mice were killed at 10 days. Numbers in brackets are standard deviations.

Table 2. Donor content of bone marrow stroma from radiochimeras

	% Donor GPI GPI-1A→1B*			GPI-1B→1A*		
	$\bar{x}$	S	N	$\bar{x}$	S	N
Whole bone marrow at 22wk post radiation	91.8	2.95	5	94.8	1.7	5
Stroma after 2wk culture	76.8	8.7	4	66.9	10.8	4
At 1st passage (4wk)	68.5	3.4	4	68.0	9.5	4
At 2nd passage (6wk)	67.3	8.5	3	66.3	8.7	3
At 3rd passage (8wk)	64.3	13.6	2			

N refers to the number of mice from which the result was obtained. S is the standard deviation; * These headings indicate the donor and recipient GPI phenotype.

Table 3. Fractionation of cultured stromal cells with carbonyl iron

	Total cells	Fe fraction	Non Fe fraction
Cell/flask ($\times 10^6$)	4.3 (1.7)	1.0 (0.45)	3.3 (1.4)
Specific activity of GPI (mU/10^6 cells)	4 (1.1)	11 (4.3)	3.5 (0.9)
% Donor GPI	85	91* (9.5)	70* (12.2)

5 culture flasks were compared after 2 weeks of culture, 1 from each of 5 chimeric mice. Numbers in brackets are standard deviations. * The iron fraction contained significantly more donor GPI than the non iron fraction ($p < 0.05$ using Student's paired t-test).

tures failed to proliferate due possibly to a low seeding rate. 2 weeks after the cultures were set up, a closely woven network of epithelioid cells formed around open areas usually containing round granular macrophage-like cells. These macrophage-like cells had 1 or 2 bean-shaped nuclei, were acid phosphatase (fig.) and non-specific esterase positive and phagocytosed opsonized sheep red blood cells. The epithelioid cells were negative for the 2 enzymes and did not phagocytose. They had branching processes and pale staining nuclei with 2 nucleoli. Fat cell colonies were often formed at about 4 weeks.

Cell fractionation was carried out in an attempt to determine whether epithelioid and macrophage-like cells corresponded to the recipient and donor GPI contributions. Phagocytosis of carbonyl iron[6] was used to separate the phagocytic populations. The results are shown in table 3. Cells from stromal cultures that had taken up carbonyl iron were 91% donor GPI whereas cells remaining after treatment were 70% donor GPI. Some of this latter donor GPI was due to macrophage-like cells that were not extracted by this regime but despite the higher GPI specific activity of the iron fraction, residual macrophage-like cells would probably not account for all the donor GPI in the non-iron fraction. Friedenstein et al.[7] and Golde et al.[3] have concluded that bone marrow fibroblasts from mice and men are recipient in origin in radiochimeras. However, in vitro establishment of hematopoietic cultures using these cells as the microenvironment was not described. Keating et al.[2] claimed that the in vitro microenvironment was donor in origin in a study of cultured bone marrow from human patients treated with cyclophosphamide, radiation and bone marrow transplant.

Thus, while it appears that there is a mesenchymally derived cell that may not be replaced by bone marrow transplant, the question remains is this the cell that permits hematopoiesis in vitro. Our results indicate that a substantial part of stroma of mouse bone marrow cultures is donor derived. That it is also derived from a hematopoietic stem cell is suggested by our failure to detect any evidence of hematopoiesis or transfer of donor cells from primary stromal cultures (table 1). In vivo in the chimera colonization of the mechanocytic framework of the host by new donor-derived microenvironment-macrophagic and epithelioid cells may take some time to manifest itself.

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External strontium and contractility in single giant muscle fibers of the barnacle

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Summary. Isometric tension and membrane potential in response to electrical stimulation have been studied on single giant muscle fibers of the barnacle upon replacement of external calcium with strontium. Under these conditions, the membrane response showed after the normal peak depolarization, a plateau phase lasting 3–7 sec before repolarization took place, while the force of contraction showed a linear relation with external strontium concentration. A direct action of strontium ions on contractile proteins (namely troponin) can be ruled out in favor of a triggering action on the sarcoplasmic reticulum, which, in turn, leads to calcium release and development of tension.

In crustacean muscle fibers the activation of membrane Ca^{2+} channels, and the resulting inward Ca^{2+} current, normally initiates the electrical response[3,4] which represents the first step in the series of events leading to contraction[5]. However, in these fibers Ca^{2+} channels do not seem to be very selective for Ca^{2+}. Hagiwara and Naka[6] obtained action potentials in EDTA-treated fibers even when the external Ca^{2+} was completely replaced by Sr^{2+}, suggesting that this ion can pass through the membrane, probably through the channels carrying the inward current. Further studies using the voltage clamp technique have shown that the amplitude of the inward currents for Ca^{2+} and Sr^{2+}, respectively in Ca- and Sr-media, became maximal at the same membrane potential level, and their ratio was of the order of unity[7]. These reports have suggested the present work, which I have carried out to study the effect of replacement of external calcium with strontium on isometric tension and membrane potential responses in electrically stimulated single muscle fibers.

Methods. The experiments were done on single giant muscle fiber from the barnacle *Balanus nubilus.* These were isolated and cut at one end, and then firmly attached to a glass cannula through which the stimulating-recording electrode[8] was inserted. The fibers were set up vertically with conventional arrangements for recording tension with the RCA 5734 transducer. Tension output was in the range 1–10 Newton · cm^{-2} (2–20% T_{max}) to avoid the fibers being pulled away from the cannula. Isometric tension and membrane potential signals were displayed on a Tektronix storage oscilloscope, where the records could be readily photographed. The EGTA solutions were injected axially inside the fiber with a microsyringe having a glass capillary (100–120 μm diameter) attached to the stainless-steel needle. Details of the dissection, cannulation and experimental apparatus may be found in Ashley and Ridgway[8]. The artificial sea water (ASW) used contained (mM): NaCl 510.4, KCl 12.9, $MgCl_2$ 23.6, $CaCl_2$ 11.8, $NaHCO_3$ 2.6 and 2 mM-TES; pH was 7.25.

Results and discussion. In an attempt to understand to what extent Sr^{2+} can substitute for Ca^{2+} in the mechanism of contraction, isometric tension in response to test stimuli was recorded on fibers bathed in ASW, and after replacement of the solution either with 0Ca-ASW or 12 Sr-0Ca-ASW Neither in 0Ca-ASW nor in 12 Sr-0Ca-ASW was the resting membrane potential significantly changed, the average depolarization observed being 2–3 mV[9]. The