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SHORT COMMUNICATION

Tridimensional networks of haematopoietic stromal cells from trout kidney formed in fibrin clots

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The present communication demonstrates that the stromal cells isolated by long-term culture of adherent cells from rainbow trout pronephros are able to form haemotopoietic stroma-like tridimensional structures when cultured in fibrin clots.

The subcultures of pronephric stromal cells used in this study have been previously described and their cells identified as stromal and capable of undergoing spontaneous haematopoiesis under special conditions (Diago, 1990). Subcultures of stromal cells consisted of monolayers of two main adherent cell types. One of the types, formed stellate, non-phagocytic, fibroblastic-like cells, resembling structurally and histochemically the reticular cells forming the network of the haematopoietic tissue (Diago *et al.*, 1991). The other cell type consisted of large, rounded epitheloid cells, showing numerous vesicles in the cytoplasm and exhibiting characteristics of the sinusoidal cells which line the renal blood sinusoids *in vivo*. These cells and endothelial cells are suspected to be *in vivo* targets of salmonid rhabdoviruses (Diago *et al.*, 1993), their destruction probably contributing to the haemorrhages characteristic of these diseases.

Adult trout (180–250 g), *Oncorhynchus mykiss*, were purchased from a commercial fish farm (Los Leoneses, Castrillo del Porma, León). After tests indicated they were free of infectious pancreatic necrosis virus, fish were maintained in 400 l tanks supplied with running, dechlorinated, pathogen-free water at 15° C ± 1° C. Culture medium consisted of RPMI-1640 containing 25 mM HEPES buffer (Gibco, Grand Island, NY, U.S.A.), supplemented, in addition to normal constituents, with L-glutamine (2 mM), sodium pyruvate (2·5 mM), the four nucleosides (25 mM each), 2 β-mercaptoethanol (5 μM), gentamycin (100 $\mu g\ ml^{-1}$), fungizone (2 $\mu g\ ml^{-1}$), 10% fetal calf serum (FCS) and 5% rainbow trout pooled sera. The pH of the medium was adjusted to 7·4 and the osmolarity to 295 mosmol kg^{-1}. Trout were anaesthetised with 0·05% tricaine methanesulfonate (MS-222, Sandoz) exsanguinated from the caudal vein and the head kidney (pronephros) removed under sterile conditions. Pronephros fragments of 1 mm^3 were washed in 0·05 M sodium phosphate, 0·15 M sodium chloride, pH 7·2 (PBS) and then incubated in 24-well culture plates (Costar, Cambridge, MA, U.S.A.) with 2 ml of culture medium at 18° C in a humidified air atmosphere. Cell monolayers were detached by exposure to 0·05% trypsin, 0·02% EDTA in 0·85 g l^{-1} NaCl aqueous solution for subculture. Cell suspensions were centrifuged at 300 *g* at 4° C for 10 min, resuspended

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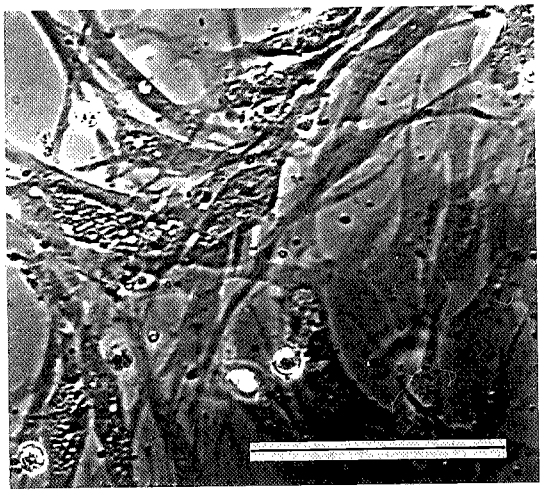

Fig. 1. Phase contrast morphology of monolayers of stromal cells in flask liquid culture after 9 months (9 passages). Bar=100 μm (× 330).

in 5 ml of culture medium at 1×10^4 cells ml^{-1}, and plated onto 25 cm^2 flasks (Costar). Subcultures were incubated as indicated above. Pronephric stromal cells were obtained from 9-month subcultures. This period of culture was necessary to obtain enough cells (nine passages, one passage per month) to perform several experiments. Fibrin clot stromal cell cultures were made by including 0·2 mg fibrinogen ml^{-1} (A. B. Kabi, Stockholm, Sweden) in the medium and 2–4 National Institute of Health Units thrombin ml^{-1} (Miles, Elkhart, IN, U.S.A.) in the wells. Fibrin-clots formed shortly after pipetting the cell containing medium into the wells. After culture, clots were flattened onto glass slides, fixed, stained with toluidine blue and cells counted in the microscope at 100 × with the aid of an ocular eye piece as described (Rueda & Coll, 1988; Coll, 1993; Estepa & Coll, 1992, 1993*a*,*b*).

Figure 1 shows the appearance of the trout stromal cell monolayers used for the fibrin clot cultures after 9 months passage. Figure 2(a) shows the trypsin-digested stromal cells plated at time 0 in fibrin-clot cultures. They appeared as an homogeneous large round cell population of about 40 μm in diameter with large nuclei. When cultured for 1 week, the number of cells did not increase significantly (44 000 cells/well to 51 600 cells/well with about a 20% coefficient of variation). However, they were observed to produce projections which connected each other and formed a tridimensional net as shown after flattening of the tridimensional fibrin clot cultures [Fig. 2(b)]. No morphological differences were apparent among the cell population that formed the tridimensional net, however, further studies need to be done to demonstrate its probable heterogenicity.

There are few *in vitro* studies using stromal cell cultures from the trout haematopoietic tissue and this report is the first to show that the tridimensional morphology of the trout kidney stroma can be reconstituted *in vitro*. Although it is difficult to see such a structure in the histological studies (Castillo *et al.*, 1987), it has been assumed that the stromal cells form a tridimensional network in which most haematopoiesis occurs in trout (Zapata, 1979) and mammals (Dexter & Spooncer, 1987; Cumano *et al.*, 1992). Since the subcultures of the pronephric stromal cells can support haematopoiesis (Diago, 1990), the growth of the stromal cells in fibrin clots resembles both the haematopoietic and the tridimensional stromal microenvironment. The presence of an intact microenvironment seems to be a requisite for *in vivo* haematopoiesis in higher vertebrates (Dexter & Spooncer, 1987) and probably in fish (Castillo *et al.*, 1987; Zapata, 1979), therefore, more extensive characterisation studies on the trout

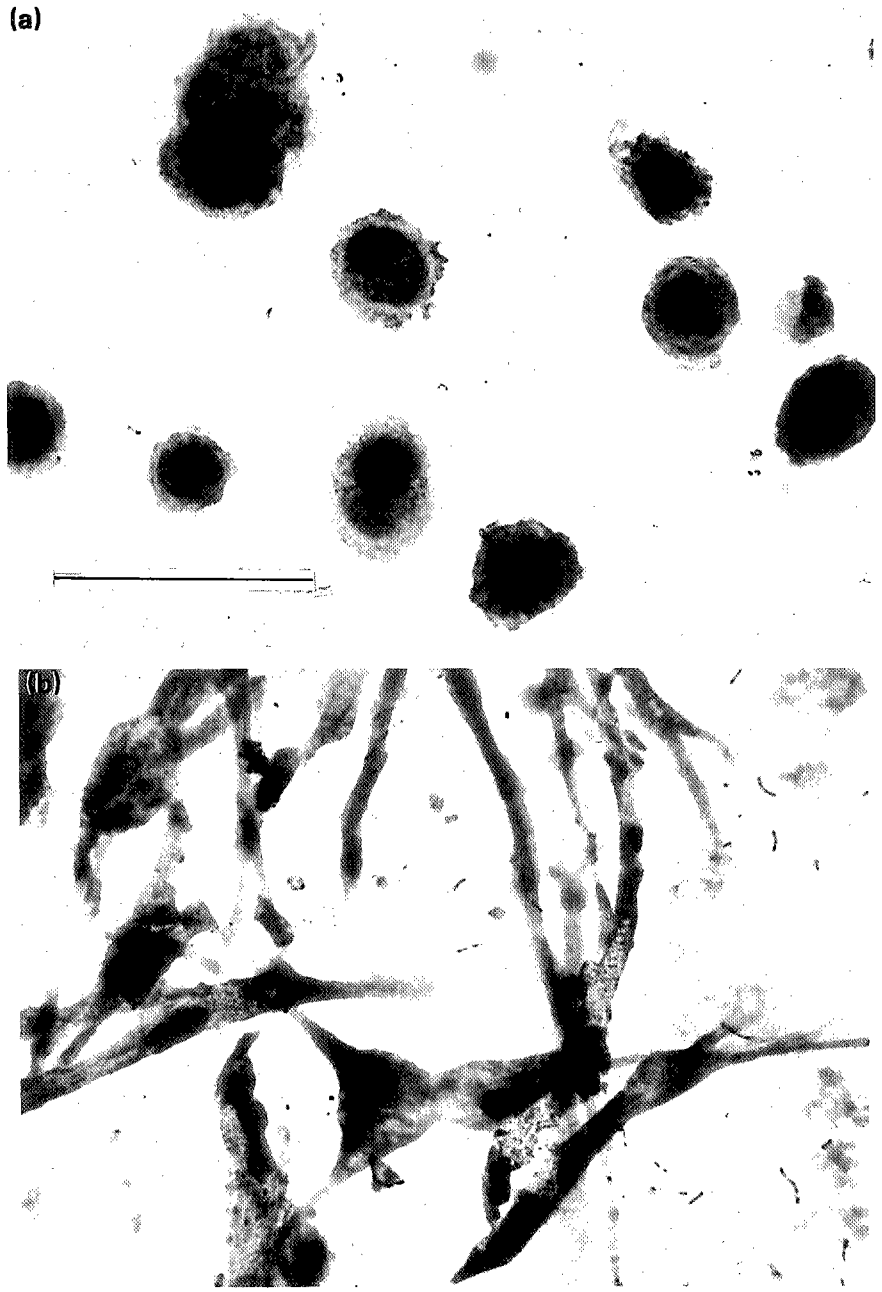

Fig. 2. Morphology of the stromal cells in fixed fibrin-clots. Recently trypsinized stromal cell monolayers (a) or tridimensional cell networks (b) in fibrin clots were flattened to glass slides, fixed and stained. Recently trypsinised stromal cell monolayers at time 0 (a). After 1 week at 18° C (b). Bar=100 μm (× 330).

haematopoietic cell populations morphologically resembling the *in vivo* situation can now be performed.

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