A nude mouse xenograft model of fetal intestine development and
differentiation

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Summary

This report describes a novel in vivo model of intestinal differentiation. Fourteen day, undifferentiated fetal rat small intestine, stripped of the major part of its mesenchyme, suspended in a type I collagen gel and then xenografted into a nude mouse, undergoes small intestinal morphogenesis and cytodifferentiation. All four major epithelial lineages, namely Paneth, goblet, columnar and endocrine are present. Double-label non-isotopic in situ hybridization, employing biotinylated and digoxigenin-labelled whole rat DNA and whole mouse DNA probes, was performed to distinguish donor cells from host cell types. The outer longitudinal smooth muscle layer, and the major part of the lamina propria, including pericryptal fibroblasts, are of host mouse origin; the inner circular smooth muscle layer is of donor rat origin. Cells of the muscularis propria and lamina propria acquired smooth muscle α-actin, presumably under the influence of the donor endoderm. Furthermore, this xenograft develops a host vascular network, and cells with the morphological appearance of lymphocytes are present within the intestinal epithelium. The production of chemotactic factors by the endoderm is postulated because grafting of collagen gel alone results in a minimal invasion by stromal cells which do not express smooth muscle α-actin.

Key words: intestinal development, epithelial-mesenchymal interactions, xenograft, collagen.

Introduction

The study of the mechanisms underlying developmental and differentiation processes requires adequate in vitro and/or in vivo model systems. Normal intestinal epithelial cells may be maintained in vitro for only short periods of time and a major problem exists in maintaining their growth and differentiation (Kedinger et al., 1987). These problems have been overcome, to a certain degree, by organ culture techniques (Altmann and Quaroni, 1990; Arsenauld and Menard, 1985; Fukamachi et al., 1986; Ishizuya-Oka and Mizuno, 1984) and establishing various intestinal tumour cell lines (Zweibaum et al., 1985; Whitehead et al., 1987; Kirkland and Bailey, 1986). Organ culture allows only short-term studies before tissue viability and/or loss of tissue architecture becomes a limiting factor. Tumour cell culture studies have yielded useful information relating to the differentiation of intestinal tumour cells: for example, some cell lines undergo morphological differentiation when cultured in various extracellular matrix proteins or on mesenchymal cellular elements (Kirkland, 1988; Richman and Bodmer, 1988; Agrez, 1989; Fukamachi and Kim, 1989; Del Buono et al., 1991a). However, intestinal tumour cell lines do not retain all of the characteristics of normal intestinal cells, and therefore do not reflect all normal cell functions. Another major problem with studies in vitro is the inability to recreate fully the in vivo environment. Consequently, to avoid the problem of cultures in vitro, models of intestinal development and differentiation have been developed where cells or tissues are maintained in vivo. The pioneering work of Le Douarin and colleagues (1968), who showed that embryonic chick fore-gut endoderm differentiated when grafted into the mesenchyme of the lateral plates of chick embryos, led subsequently to the establishment of in vivo models of epithelial-mesenchymal interactions in gastrointestinal differentiation. These models, which gave rise to well developed intestinal units, were based on avian, rodent and human homospecific and hetero-specific epithelial cell and mesenchymal cell recombinants, grafted into chick embryos in ovo, (Gumpel-Pinot et al., 1978; Kedinger et al., 1981). In addition,
typical gastrointestinal organogenesis occurs when appropriate endoderms and mesenchymes are associated and grafted under the renal capsule of rodents (Kedinger et al., 1983).

Recently, using a novel xenograft system, the importance of epithelial-mesenchymal interactions in intestinal tumour differentiation has been emphasised (Del Buono et al., 1991b). A rectal adenocarcinoma cell line precultured in a type I collagen gel and then subcutaneously xenografted into nude mice, differentiates in response to mouse host connective tissue cells migrating through the gel. The aim of the work reported here is to use this novel xenograft system to develop a simple and convenient in vivo model of intestinal morphogenesis and differentiation. We show that normal undifferentiated fetal rat intestinal endoderm is induced to undergo differentiation as small intestine when suspended in a type I collagen gel and then xenografted into nude mice. Furthermore, using double non-isotopic in situ hybridization for mouse and rat DNA to distinguish between host and donor cell nuclei we show that this endoderm/collagen xenograft acquires a stroma derived in major part from the mouse host, and discuss further the potential of such xenografts as simple and convenient models of intestinal development and differentiation.

Materials and methods

Animals

Fourteen day pregnant Wistar rats were provided by Clare Hall Laboratories, ICRF, South Mimms, Herts. UK. The existence of a vaginal plug was used to designate day 0. Dams were anesthetized by Halothane (ICI, Macclesfield, Cheshire, UK) inhalation. The fetuses were delivered by Caesarean section and the presumptive small intestines removed into sterile physiological saline.

Young adult outbred Swiss athymic immunodeficient (nu/nu) mice (Clare Hall Labs.) were used as recipient hosts. All animals were fed ad libitum.

Type I collagen gel

Type I collagen was prepared from rat tail tendons as previously described (Schor, 1980). A working solution of 0.3% collagen in 10mM acetic acid was used and neutralized immediately prior to use by addition of 10× basal Eagle's medium (Gibco, Paisley, Scotland, UK) and 0.4M NaOH (2:1 mixture, respectively), dropwise, until pH 7 was reached.

Dissociation and grafting procedure

Small intestinal anlagen were incubated with 0.03% collagenase IV (Worthington Biochemicals Corp., NJ, USA) in CMRL 1066 medium (Gibco), for 45 minutes at 37°C, as described by Gumpel-Pinot and colleagues (1978). Thereafter, the mesenchyme was dissected away from the endoderm under a dissecting microscope. Neutralized collagen (1ml) was poured into 35mm Falcon tissue culture dishes. Eight endoderm fragments (2-5mm lengths) were suspended at regular intervals throughout the collagen, which was then placed for 15 min in an incubator containing 10% CO2 at 37°C, to allow gelation. The collagen was then cut into four segments containing two endoderm fragments, each segment being xenografted into the flanks of nu/nu mice (see Fig. 1), as described previously (Del Buono et al., 1991b). All procedures were performed on the same day. Endoderm fragments were also cultured in type I collagen in vitro, in medium consisting of 15% fetal calf serum (fcs) (Gibco), 85% CMRL 1066 and 200μg/ml gentamycin. Endoderm cultures were terminated by addition of neutral buffered formalin (nbf) when the first signs of cell lysis were apparent, and xenografts were harvested after 10 days, fixed in nbf processed through graded alcohols and embedded in paraffin wax. Sections (4μm) were stained with Haematoxylin and Eosin (H & E), Alcian blue-Periodic acid-Schiffs (AB-PAS) and Grimelius silver stain; immunocytochemistry and in situ hybridization was also performed on 4μm paraffin sections.

Fig. 1. Schematic diagram outlining the grafting procedure.
Fig. 6. Double-label in situ hybridisation. Rat genomic DNA probe, nick translated with biotin-11-dUTP, was detected with avidin-alkaline phosphatase and visualised using NBT/BCIP substrate, producing a purple precipitate. Mouse genomic DNA probe, nick translated with digoxigenin-11-dTUP, was detected with anti-digoxigenin-peroxidase conjugate and visualised using AEC substrate, producing a brown precipitate. (A) A villus showing rat derived epithelium (purple) and the majority of villous core cells of mouse origin (brown). A cell with the morphological appearance of a lymphocyte, of mouse origin, is also visible (arrow). (B) Outer longitudinal muscle layer (l) of mouse origin (brown) and inner circular muscle layer (c) of rat origin (purple). (C) Pericryptal fibroblasts of mouse origin (brown). Bar=25μm.
In situ hybridization

In situ hybridization was performed essentially as described previously (Porter et al., 1988) with modifications to allow simultaneous detection of DNA from both donor and host species in a single section, so as to distinguish between rat and mouse derived tissue. After proteolytic digestion with proteinase K (10-20 µg/ml in 300 mM sodium acetate, 200 mM Tris, 50 mM EDTA, 1% SDS, 1% of lauroyl sarcosine pH 9.0) at 55°C for 10-15 minutes, denatured sections were incubated with hybridization mixture containing biotinylated whole rat DNA probe recognising rat sequences and digoxigenin labelled whole mouse DNA recognising mouse sequences, both at concentrations of 2 µg/ml. Additional high stringency washes in 0.5 x PBS at 65°C were performed after hybridization. Bound probes were detected with avidin-alkaline phosphatase conjugate (1:250) and anti-digoxigenin-peroxidase conjugate (1:30) applied simultaneously. The peroxidase signal was developed first using 3-amino-9-ethylcarbazole (AEC) substrate (Herrington et al., 1989) (producing a red-brown precipitate in nuclei of mouse origin) and the alkaline phosphatase was visualised with nitrobluetetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT-BCIP) substrate, producing a purple precipitate in nuclei of rat origin. The specificity of the probes was checked on control tissues from rat and mouse. Under high stringency conditions, there was minimal cross-reactivity.

Immunocytochemistry

Dewaxed, paraffin-embedded sections were stained with a monoclonal antibody to smooth muscle α-actin (Sigma, St. Louis, MO, USA), by the indirect peroxidase method.

Results

Fourteen-day fetal rat intestine

At 14-days gestation, the fetal rat small intestine is morphologically and cytologically undifferentiated and consists of a simple tube composed of radially arranged stratified endodermal cells, surrounded by a mass of undifferentiated mesenchymal cells (Fig. 2).

Endoderm-collagen cultures

Collagenase treatment of fetal rat intestine allowed mechanical separation of the major part of the mesenchyme from the endoderm. However, few mesenchymal cells were apparent, under phase contrast microscopy, in patches along the length of the endoderm (Fig. 3). Within 24h in collagen, the mesenchymal cells had covered the length of the endoderm. Small buds were observed protruding from the endoderm rod at 24h and these progressively elongated forming a branched structure (Fig. 4A). After 5 days, cell lysis was observed and the culture terminated. On histological examination, there was a loose mesenchymal element surrounding a tube of morphologically undifferentiated endoderm (Fig. 4B). The protrusions were outgrowths of the mesenchymal element, and endodermal cells were not present within these protrusions.

Endoderm-collagen xenograft

Endodermcs suspended in a type I collagen gel and xenografted into nude mice, give rise to a well differentiated small intestinal morphology within 10 days. Well-developed crypts and villi contain the four cell types known to be present in the small intestine, namely absorptive columnar, Paneth, goblet and entero-endocrine cells. Furthermore, the graft develops a stroma consisting of well formed circular and longitudinal smooth muscle layers and a lamina propria (Fig. 5).

The specific origin of cells was deduced by double-labelling in situ hybridization, which allowed simultaneous detection of DNA from both rat donor and mouse host species. The epithelium and the circular smooth muscle stain purple and are thus of rat origin. Longitudinal smooth muscle layer and the vast majority of lamina propria connective tissue cells, pericryptal fibroblasts and intraepithelial lymphocytes are of mouse origin (see Fig. 6).

The distribution of smooth muscle α-actin was localised to the muscularis propria, cells in the villous core and pericryptal fibroblasts (Fig. 7).

Type I collagen grafted alone and harvested up to four weeks later contained very few stromal cells which did not express smooth muscle α-actin (Fig. 8).

Discussion

This report describes a novel xenograft system in mammals to study intestinal development and differentiation in vivo. Fetal rat intestinal endoderm embedded
in type I collagen gel and transplanted into the subcutaneous tissue of nude mice differentiates as small intestine. Double-label non-isotopic in situ hybridization was performed to distinguish between host and donor nuclei, revealing an interesting juxtaposition of rat and mouse cells. Host stromal cells migrate through the gel to subsequently form the bulk of the lamina propria and longitudinal muscle layer. Blood vessels enter the graft, and host cells with the morphological appearance of lymphocytes are clearly visible within the intestinal epithelium. The relatively few donor mesenchymal cells remaining attached to the endoderm during the dissociation procedure, form the circular smooth muscle layer. This juxtaposition of rat donor and mouse host stromal cells remains sorted in distinct layers of the developing intestinal tissue. Furthermore, these stromal cells are induced to express $\alpha$-actin, presumably by the epithelium.

This model of xenogeneic grafts between two species of rodents has certain advantages compared with available techniques. It is more physiological than xenoplastic recombinants between embryonic chick and rodent tissues grafted in ovo (Kedinger et al., 1981). The use of nude mice as recipients allows the study of the development and differentiation of the graft for long periods of time without immune rejection. From a practical viewpoint, this subcutaneous xenograft system is a far simpler and more convenient technique compared with intracoelomic grafting into chick embryos or intrarenal capsule grafting into rodents. Furthermore, the surgical insult to the animal is reduced considerably compared with intrarenal capsule grafting.

In addition, this report presents the first use of double-label in situ hybridization for the differentiation of cells of different species origin in single tissue sections. A similar strategy has previously been utilised for the non-isotopic co-detection of human and viral sequences (Herrington et al., 1989) or sequences from two viruses (Mullinck et al., 1989) in single cells. The availability of an increasing range of different labelling options provides a very flexible means of detecting multiple nucleic acid targets in cellular preparations (Morey et al., 1991), and offers a simple and reliable method for determining the origins of cells in xenograft systems as described here.

The importance of epithelial-mesenchymal interactions in the morphogenesis of the gastrointestinal tract is well established. Two classes of such interactions have emerged from experimental studies, based on inductive properties exerted by the mesenchyme on endoderm differentiation: instructive and permissive. An instructive inductive effect triggers a new develop-
mental programme specified by the mesenchyme. This was first demonstrated in associations of chick intestinal mesenchyme and stomach endoderm, which, when grafted into the coelomic cavity of chick embryos, gave rise to an intestinalised epithelium (Haffen et al., 1982).

A permissive inductive effect triggers a previously determined developmental programme specified by the endoderm. Chick intestinal mesenchyme and rat intestinal endoderm associations grafted in the coelomic cavity of chick embryos give rise to an intestinal epithelium and the pattern of expression of brush border enzymes corresponded to that of the species of origin of the endoderm. (Kedinger et al., 1981).

Clearly then, contamination of transplanted tissues with permissive or instructive cell types may confound the interpretation of results. The purity of collagenase IV dissociated endoderm and mesenchyme in most of the studies on epithelial-mesenchymal interactions was confirmed by Le Douarin's marker technique (Le Douarin, 1973; Haffen et al., 1987; Haffen et al., 1981) based on the difference in nuclear structure of two avian species. In our hands, however, completely mesenchyme-free endoderm was not achieved - a relatively small number of mesenchyme cells remained attached during the dissociation procedure, as seen in the collagen cultures in vitro. This was further confirmed by double-label in situ hybridization on the 10-day xenograft which revealed an unusual juxtaposition of rat donor and mouse host-specific stroma. These observations suggest that the original mouse-derived stromal cells, which subsequently form the lamina propria cells, migrated through the rat-derived inner circular smooth muscle layer. Furthermore, there is very little mixing of host and donor stromal cells - there are definite bands of species specific smooth muscle layers. This implies a sorting phenomenon. It is clear that the donor mesenchymal layer expanded in all directions, as it did in the collagen cultures in vitro, before differentiating. It is known that fibroblasts undergo a fixed (programmed) number of cell divisions in vitro before dying (Hayflick, 1968). It is not unreasonable to suggest, then, that these mesenchymal cells may already have been committed to circular smooth muscle differentiation, and hence are not involved in the formation of lamina propria or longitudinal smooth muscle layer.

A permissive induction of intestinal endoderm by heterologous mesenchyme has been reported previously (Haffen et al., 1983). Fetal lung or skin fibroblasts, associated with chick embryonic gizzard and grafted in the coelomic cavity of chick embryos, gave rise to gizzard epithelium. The endoderm in turn had an inductive influence on the fibroblast cells, which differentiated to smooth muscle layers. Because of the mixed population of stromal cells, it is not possible at present to say which population had an inductive influence on the differentiation of the endoderm. Furthermore, the site of origin of the mouse stromal cells is not clear. What is apparent is that these stromal cells do not originally express smooth muscle a-actin but are induced to do so by the epithelium. Two lines of evidence support this: in a previous report, a rectal adenocarcinoma cell line, precultured in a type I collagen gel and then xenografted, underwent glandular differentiation and acquired a host-derived stroma (Del Buono et al., 1991b). Expression of a-actin was localised to a band of stromal cells, 4-6 cells thick, adjacent to and around the glandular epithelium. Stromal cells distal to the epithelium did not express a-actin (unpublished observation, Del Buono). The second line of evidence comes from the grafting of collagen alone. The few stromal cells that did invade did not express a-actin.

The involvement of host stroma in xenografts of tumour cells is well established (Giovanella and Fogh, 1985). Xenografted tumours elicit, from the host, a stroma complete with vascular bed and fibrous tissue for support. Thus neoplastic tissue is able to secrete diffusible angiogenic or chemotactic factors capable of attracting connective tissue cells. Our observation that collagen gel grafted alone for up to four weeks contained few host cells would suggest that the grafted endoderm, or indeed the contaminating mesenchymal cells, "attracted" the host stromal cells, possibly by releasing a chemotactic factor. Indeed, extracellular matrix proteins such as interstitial collagens (I and III), elastin and fibronectin are known to be chemotactic for...
Fig. 7. (A) Villous core cells (bar=30μm) and (B) pericryptal fibroblasts (bar=15μm), both of mouse origin, expressing smooth muscle α-actin.

mesenchymal and other cells in vitro (McDonald, 1989), as are the multipotent regulatory proteins, transforming growth factor beta (TGFβ) (Sporn and Roberts, 1988), and tumour necrosis factor (TNF) which are also able to stimulate the production of connective tissue (Kovacs, 1991).

The exact role of the collagen in the differentiation process of the endoderm is not clear. Collagen does not seem to have any effect on morphological differentiation of the endoderm in vitro. Subcutaneous xenografting of endoderm alone (ie. without the collagen) was performed on several occasions, but on harvesting, it could not be located. The problem is that 14-day fetal rat endoderm is 40-50μm wide, barely visible to the naked eye. Attempts made at “marking” the endoderm with charcoal powder to facilitate location were unsuccessful.

The role of collagen in a xenograft system has been reported previously (Del Buono et al., 1991b). In vitro collagen-induced glandular structures of a rectal adenocarcinoma cell line when xenografted in nude mice differentiated in response to invading host stromal cells. Furthermore, the time taken to form a tumour was dramatically reduced in this cell-collagen xenograft. Type I collagen represents 80 per cent of the total collagen synthesised by stromal cells (Kovacs, 1991). The existence of a collagen gel may, therefore, confer a growth advantage upon such xenografts. In the presence of type I collagen, stromal cells might not be required to synthesise and deposit collagen. Thus, the type I collagen gel may simply behave as a “medium”

Fig. 8. Collagen gel grafted alone into nude mice. Relatively few stromal cells are present. H and E. Bar=25μm.
allowing the movement of cells and diffusion of humoral factors.

In conclusion, this simple and convenient in vivo model system will be useful not only for the study of epithelial-mesenchymal interactions in intestinal development and differentiation, but also for the study of chemotactic factors responsible for the migration of host stromal cells in the collagen cell, the commitment of mesenchymal cells, the fate of hemopoietic cells which migrate inside the intestine rudiment, and will allow manipulation of endoderm cells to study crypt genesis and cell lineage in the developing gastrointestinal tract. Furthermore, the novel application of double non-isotopic in situ hybridization for species-specific DNA offers a simple and reliable means of determining the origins of cells in such model systems.

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References


