Beige granules as a cell marker for clonal analysis in kidney and liver of mouse aggregation chimaeras, and three-dimensional reconstruction from serial paraffin sections

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Summary
Anomalous giant granules of beige (bg) mice have been used as a cell marker in the study of cell lineage of mast cells. Similar granules are known to exist in other tissues including kidney proximal tubules and liver parenchymal cells. In the present study, these granules were found to give yellow or orange autofluorescence when the tissue had been fixed with formaldehyde and embedded in paraffin. Thus, the granules can be used as a cell marker that can be visualized in serial paraffin sections without any specific histochemical staining. Chimaeric mice were produced by aggregation of 8-cell-stage embryos of beige (C57BL/6J-bg/J/bg/J) and A/J strains. The chimaeric liver showed beige cell patches with complicated shapes, although the patches frequently conformed to the shape of parenchymal cell cord or plate structures. In chimaeric kidney, beige cells formed coherent patches in the proximal tubules. The tubules were found to contain more than one clone. The patches frequently had long extended shapes suggesting growth of the clone along the tubule axis. Three-dimensional image reconstruction from the serial paraffin sections was carried out with the aid of a computer-assisted image analysis system, resulting in a clearer image of the patch shape.

Key words: chimaera, clonal analysis, mouse, beige mutation, kidney proximal tubule, liver parenchyma.

Introduction
Chimaeric animals have been used for the analysis of cell lineage and clonal cell patches in various mammalian tissues (McLaren, 1976). The most important prerequisite in such studies is availability of the cell marker (West, 1984; Iannaccone, 1987). An obvious cell marker such as pigmented versus unpigmented strains has been used for analysis of the coat colour pattern and histogenesis of other pigmented tissues (McLaren, 1976).

Other cell markers available for mammalian tissues, such as enzyme mutations or H-2 antigens (Ponder et al. 1983), require elaborate histochemical staining procedures, such as immunostaining (Ponder et al. 1983, 1986; Weinberg et al. 1985), enzyme histochemistry (Dewey et al. 1976; West, 1976; Herrup et al. 1984; Mullen, 1978), or in situ DNA hybridization (Rossant, 1985). In these cases, cryosectioning is the most frequently used method to obtain tissue sections. Combination of these two procedures inevitably makes it very laborious and time-consuming or, sometimes, practically impossible to obtain undisrupted serial sections for analysis of three-dimensional tissues.

In fact, the most successful clonal analyses so far used two-dimensional analysis if the particular tissue can be unfolded and spread into flat sheets (Ponder et al. 1986; Schmidt & Ponder, 1987). The following tissues have been studied: the intestine epithelium (Schmidt et al. 1984, 1985a,b; Ponder et al. 1985), blood vessel walls (Schmidt et al. 1986b), retinal pigmented epithelium (Schmidt et al. 1986a; Bodenstein & Sidman, 1987; Hunt et al. 1987a,b) and extraembryonic membranes (Gardner, 1984, 1985). However, many tissues cannot be spread into two-dimensional sheets. In most cases, intermittent sections were examined and hypothetical reconstructions made of the three-dimensional shape and distribution of clonal cell patches. Therefore, a cell marker that can be visualized by simple and reliable methods in paraffin sections is very desirable, because it is still...
Animals and embryos

Granules in other tissues except pigmented cells have been visualized with specific dye staining. The granules have been used as a cell marker for clonal chymal cells (Oliver & Essner, 1973). Such giant tissues, including pigment cells, mast cells, eosinophil leucocytes, kidney proximal tubules, liver parenchymal cells (Oliver & Essner, 1973). Such giant granules have been used as a cell marker for clonal analysis of mast cells (Kitamura et al. 1977, 1979) by visualizing the granules with specific dye staining. The granules in other tissues except pigmented cells have been visualized by enzyme histochemistry for acid phosphatase or by transmission electron microscopy. Such studies suggested that the granules are lysosomes and related organelles (Oliver & Essner, 1973).

The present study shows that the giant granules in the kidney proximal tubule cells and liver parenchymal cells can be visualized by their autofluorescence when the tissue is fixed with formaldehyde and processed for ordinary paraffin embedding and sectioning. Thus, in tissues from chimaeraic mice between beige and normal strains, the beige cell can be recognized in ordinary paraffin sections without any specific or elaborate staining. Furthermore, use of a computer-assisted image analysis system enabled three-dimensional image reconstruction of beige cell patches in the liver parenchyma and kidney proximal tubules from serial paraffin sections.

Materials and methods

Animals and embryos

C57BL/6J-bg/bg strain mice from Jackson Laboratories (Bar Harbor, USA) and A/J strain mice from Shizuoka Laboratory Animal Center (Hamamatsu, Japan) were kept under the 12 h-light (7 a.m. – 7 p.m.) and 12 h-dark cycle. Females, 7–9 weeks old, were induced to superovulate by flushing oviducts with PB1 solution (Whittingham, 1969). The zona pellucida was dissolved by treating embryos with 0–5% pronase in PB1. A pair of beige and A/J embryos were transferred into a drop of M16 medium (Whittingham, 1971) supplemented with 20% fetal bovine serum on the bottom of a plastic culture dish overlaid with paraffin oil. Two embryos were pushed together with tips of fine forceps until they adhered to each other. Then the dishes were incubated in a CO₂-incubator at 37°C with an atmosphere of 5% CO₂ and 95% air. After culture for 1 day, embryos that had developed to late morula or early blastocyst stages were transferred into uteri of the foster mothers of either pregnant or pseudopregnant ICR strain mice (purchased from Shizuoka Laboratory Animal Center).

Histology

Organs from ‘unbalanced’ chimaeras with smaller contributions from beige strain were used for clonal analysis. Visual estimation of coat colour was approximately 20–30% beige and 70–80% A/J. For the liver tissues, chimaeras with larger contribution from beige strain were also used for the analysis. Such chimaeras at 4 months of age were killed by cervical dislocation. Kidneys and liver lobes were dissected out and fixed in 10% formalin which had been neutralized with addition of CaCO₃ powder. During the fixation period of 1 day at room temperature, organs were dissected into smaller pieces. They were dehydrated through graded ethanol/butanol mixture series and embedded in paraffin.

Serial sections were cut at 10 μm of thickness in the planes either tangential (in case of kidney) or perpendicular (liver) to the organ surface. Serial sections on glass slides were treated with xylene to remove paraffin, rehydrated with PBS and finally sealed with coverslips using glycerol as the mounting liquid. A fluorescence microscope (Nikon Optiphoto) was used to examine the sections using either ultraviolet excitation (u.v. 330–380 for excitation filter and 420K for absorption filter) or violet excitation (IF395–425 excitation filter and 470K absorption filter, supplemented with O590 or R610 filters for absorption of the blue cytoplasmic autofluorescence). The violet excitation was used to obtain black and white photographs. Under u.v. excitation, the giant granules of beige cells showed yellow or orange autofluorescence. Although the cytoplasm showed blue autofluorescence, fortunately it faded much faster than the granules after approximately 1 min of bleaching with the excitation light. Therefore, all photographs were taken after bleaching the sections for 1–3 min. In fact, the autofluorescence of granules showed no recognizable bleaching during observation up to 30 min.

Three-dimensional image reconstruction

First, we took relatively low-magnification photographs of the serial sections. Then, on the photographic prints, we marked the beige cells by looking at the corresponding sections under a microscope. Such marked photographs or their traces made on transparent sheets were input into a computer-assisted image analysis system for three-dimensional reconstruction (Nikon Cosmzone-S) through a digitizer. Cross-sections of blood vessels were used as landmarks and reference points. The reconstructed images shown in Fig. 5A–D could be displayed from various angles. The images were given artificial shading to improve three-dimensional perception. Finally, different colours were assigned to the outer and inner surfaces of the structures.
Results

Liver parenchymal tissue

Fig. 1A shows a section of the liver from a beige mouse. All of the parenchymal cells contain fluorescent granules, which are absent from the kidney of A/J strain mice (Fig. 1B). The liver parenchyma cells were stained with a very simple method in serial paraffin sections. The giant granules of beige cells can be visualized with a very simple method in serial paraffin sections. Therefore, we tried three-dimensional image reconstruction of beige cell patches in the liver parenchyma and kidney proximal tubules using a computer-assisted image analysis system (Fig. 5A–D). Fig. 5A and B shows the liver tissue reconstructed from 31 serial 10 μm sections. They show a three-dimensional image of the beige cell patches, whose complicated shapes tend to be arranged radially from the central vein.

Kidney proximal tubules

Fig. 2A shows a section of the kidney cortex from a beige mouse. Cells forming the proximal tubule wall contain fluorescent granules, which are absent from the kidney of A/J strain mice (Fig. 2B). Sections of the chimaeric kidney show transverse (Fig. 2C) and longitudinal (Fig. 2D) sections of the proximal tubules which contain beige cells. In the cross-sections, some of the tubules are made of beige cells only, but many of them contain cells from both strains with variable ratios of cell number (Fig. 2C), indicating polyclonal origin of each proximal tubule. Beige cells are not dispersed among the tubules as single cells, but they form coherent patches whose shapes conform to the tubular structure. Longitudinal sections frequently show relatively large long patches of beige cells (Fig. 2D). Sometimes, one side wall of a tubule is composed of beige cells and the opposite wall is of A/J cells, suggesting the presence of patches with long slender shapes.

Fig. 3 shows tracing of the tubules containing beige cells found in one paraffin section. There is a tendency for several tubules with beige cells to cluster into larger groups. Perpendicular cross-sections of the proximal tubules were further analysed. First, cell number contained in each perpendicular cross-section was counted, with the mean and standard deviation of 8.4±1.0. Next, the percentage of beige cell number in each cross-section was calculated and its distribution is shown in Fig. 4. Tubules with small number (10–30%) of beige cells are more abundant than tubules made of nearly equal numbers (40–70%) of beige and A/J cells, probably reflecting the ‘unbalanced’ nature of the chimaera with minor contribution from beige strain. However, there is an increase in the number of tubules made almost completely (80–100%) of beige cells, probably because a beige cell clone makes a coherent patch of the same cell type and therefore has a tendency to constitute an entire segment of the tubule.

Three-dimensional reconstruction

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Fig. 5C and D show the proximal tubules reconstructed from 20 serial 10 μm sections. The beige cell patches are given a red colour for the outer surface and an orange colour for the inner surface of the tubule wall. The A/J component is indicated by a blue colour (Fig. 5C) or yellow rings (Fig. 5D). Such reconstructed images show that the patch contour is distorted and not straight. Nevertheless, one can recognize long stretched patches extending along the tubule axis, thus confirming the speculation from longitudinal sections.

Discussion

Beige mutation as a cell marker

The autofluorescence shown by anomalous giant granules of beige cells can be used as a cell marker of the liver parenchyma and kidney proximal tubules in chimaeric mice. It can be visualized in ordinary paraffin sections without any need for specific histochemical staining and it is stable under the excitation light. The autofluorescence of cytoplasm can be bleached to lower intensity within a few minutes without any recognizable bleaching of the granules. Adequate selection of filters in fluorescence microscopy allows recognition of granules even in monochrome photographs. In the preliminary studies, the granules were examined by transmission electron microscopy. They appeared as lysosomes or related multivesicular bodies as reported before (Oliver & Essner, 1973).

In order to obtain absolute evidence that all the beige cells have granules in chimaeric tissues, one must have independent double staining or markers of the same histological section. However, it would be very difficult because the granules became autofluorescent only after fixation with formaldehyde or glutaraldehyde, which most likely destroys enzyme activity or antigenicity of H-2 antigens etc. However, we observed that all the liver parenchyma cells and
Fig. 1. Fluorescence micrographs of paraffin sections showing liver lobules of beige strain (A), A/J strain (B), or chimaeric (C,D) mice. Parenchymal cells form cord or plate structures that extend radially from the central vein (v). Beige parenchymal cells contain fluorescent granules (A), which are absent in A/J cells (B). Patches of beige cells frequently conform to the cord or plate structures (C) and, thus, they tend to be radially arranged from the central vein (D). Bars, 100 µm. A–C are at the same magnification.
proximal tubule cells of the beige mouse had granules. No cells in A/J or normal C57BL/6 strain mice had granules. In chimaeric tissues, cells with typical granules exist side by side with cells without any granules; there was no intermediate cell type at the border between two cell groups. Moreover, giant granules are probably caused by an abnormality in the lysosomal membrane, and it is unlikely that normal cells affect the beige cells and eliminate the beige phenotype.

The giant granules are present in other tissues of beige mice (Oliver & Essner, 1973). Among them,

Fig. 2. Fluorescence micrographs of paraffin sections of the kidney cortex from beige (A), A/J (B) or chimaeric (C,D) mice. Cells forming the proximal tubule wall of beige mice contain fluorescent granules (A), which are absent in A/J cells (B). C shows transverse cross-sections completely (larger arrow) or partially (smaller arrows) made of beige cells. D shows many longitudinal sections of the proximal tubules. Among several patches of beige cells, arrows indicate a large extended patch which is continuous at one side wall of the tubule but disrupted at the opposite wall. g, glomerulus. Bar, 100 μm. A–D are at the same magnification.
Liver parenchymal cells
Chimaeric liver showed patches of beige cells forming complicated network in the liver lobules as reported previously (West, 1976). Three-dimensional reconstruction analysis gave clearer images of the shape and distribution of the patches. In the present study, the patches frequently conformed to the shape of parenchymal cell cord or plate structures. Such distribution probably reflects the process by which the parenchymal endoderm cells proliferate to form cord or plate structures in the fetal liver.

Kidney proximal tubules
The glomerular capsule, proximal tubule, loop of Henle and distal tubule of the metanephric nephron are formed by the metanephric mesenchymal cells after they were induced by the ureteric ampullae to form nephrogenic vesicles (Saxen et al. 1968). These vesicles undergo cell proliferation and growth, and develop into elongated tubules and glomerular capsule structures.

The present study suggests the following conclusions about the clonal history of the proximal tubules. (1) Coherent patches are formed by the tubule cells, which indicates that there is little cell mingling when the nephrogenic vesicles undergo cell proliferation and growth. (2) Each tubule is formed by more than one clone. (3) Proliferation and growth of clonal patches are greater along the tubule axis, because many patches have elongated shape along the tubule axis. (4) There are larger clustered groups of clonal patches of the proximal tubules. They probably represent 'descendant clones' as described in cases of the small intestinal epithelium and aortic endothelium (Ponder et al. 1986; Schmidt & Ponder, 1987). It means that there had been proliferation of the ancestral mesenchymal cells with some degree of cell mingling before the induction to form nephrogenic vesicles. Thus, the proximal tubules developed from those vesicles contained offspring of one particular clone and remained in the vicinity of the kidney cortex.

Three-dimensional reconstruction
Reconstruction from the serial sections revealed clearer images of the patch shapes than had been speculated from intermittent sections. Therefore, it is a useful method to study shapes and distribution of clonal patches for tissues that cannot be spread into two-dimensional sheets. However, such reconstruction would require a great amount of time and labour if there were no simple and reliable method to visualize cell markers in serial sections. In this respect, it is most desirable if the cell marker can be detected in paraffin sections, which is still the most reliable method to obtain undisrupted serial sections.

the Purkinje cells of cerebellum showed fluorescent granules which were larger in beige mice than in A/J mice. Several studies have been published on the clonal analysis of the Purkinje cells (Dewey et al. 1976; Mullen, 1978; Herrup, 1986; Herrup & Sunter, 1986) and we did not examine this tissue in the present study.

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Fig. 5. Three-dimensional image reconstruction from 31 serial 10 µm paraffin sections of the chimaeric liver (A,B), or from 20 serial sections of the chimaeric kidney cortex (C,D). Blood vessels used as landmarks are shown by green rings (A,B) or as green tubes (C,D). Bars, 100 µm. In A, three separate beige cell patches are shown by orange, red and violet colours. The red patch (B) may be made of three patches in contact. Outer surface of the liver is shown as a blue wall. In C and D, the beige cell patches are shown by red colour. The A/J component is indicated by blue colour (C) or yellow rings (D). The inner surface of the beige tubule wall is shown by orange colour, in contrast to the red-coloured outer surface. One can recognize patches with distorted contours extending along the tubule axis.
The giant granules in beige mice are such a cell marker, as shown in this study, although their presence seems to be limited to a few types of tissues. The present study warrants further utilization of the beige mutation as a cell marker.

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References


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