Macrophages in haemopoietic and other tissues of the developing mouse detected by the monoclonal antibody F4/80

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Summary

Macrophages are widely distributed in lymphohaemo-poietic and other tissues of the normal and diseased adult, where they play an important role in host defence and repair. Although the development of haemopoiesis has been well studied in several species, the ontogeny of the mononuclear phagocyte system remains poorly understood. We have used a highly specific mAb, F4/80, to examine the distribution of mature macrophages in the developing mouse, with special reference to their presence in the haemopoietic microenvironment. Monocytes and macrophages were first seen in embryos on day 10 in the yolk sac and liver as well as in mesenchyme. In liver, spleen and bone marrow, there was expansion of this population associated with the initiation of haemopoiesis on days 11, 15 and 17, respectively. Macrophages in these sites formed part of the haemopoietic stroma and their extensively spread plasma membrane processes could be seen making intimate contacts with clusters of differentiating haemopoietic cells. F4/80+ cells were widely dispersed in undifferentiated mesenchymal tissue in organs such as lung, kidney and gut. Numbers of F4/80-labelled cells increased concomitantly with organ growth and local mitoses were evident, as well as actively phagocytic macrophages. Our studies establish that macrophages are among the earliest haemopoietic cells to be produced during development and that they are relatively abundant in fetal tissues in the absence of overt inflammatory stimuli. Their distribution is correlated with the sequential migration of haemopoiesis and they constitute a prominent component of the stroma in fetal liver, spleen red pulp and bone marrow. Apart from a role in haemopoietic cellular interactions, their highly developed endocytic and biosynthetic activities suggest that macrophages contribute major undefined functions during growth, turnover and modelling of fetal tissues.

Abbreviations: Ab, antibody; ag, antigen; mAb, monoclonal antibody; MΦ, macrophage.

Key words: macrophage, haemopoiesis, mouse embryo, monoclonal antibody, F4/80.

Introduction

Although the ontogeny of haemopoiesis (Metcalf and Moore, 1971) and of lymphocyte differentiation (Owen and Jenkinson, 1981) has received a great deal of attention, development of the macrophage (MΦ) system has been neglected. Previous workers have reported the presence of MΦ in fetal tissues of several species, based mainly on morphologic and cytochemical markers, and have shown that MΦ can be derived from fetal progenitors in cell culture (for example, Cline and Moore, 1972; Deimann and Fahimi, 1978; Djaldetti et al. 1972; Naito et al. 1989).

Almost all mature MΦ populations in the mouse express a unique surface antigen (ag) defined by the F4/80 monoclonal antibody (mAb) (Austyn and Gordon, 1981; Starkey et al. 1987). The stability of this ag to glutaraldehyde fixation has allowed the identification of MΦ in the organs and tissues of adult animals with great specificity and sensitivity (Hume and Gordon, 1985). As a result, a clear picture of the distribution of MΦ in the normal and diseased adult mouse now exists (Gordon et al. 1988b). A similar comprehensive study has not yet been reported for the developing mouse. The F4/80 mab has been used to a limited extent in fetal tissues to identify MΦ in the visceral yolk sac (Matthews et al. 1985), liver and hindlimb bud (Shia, unpublished), thymus (Robinson, 1984) and urogenital ridge (De Felici et al. 1986). Earlier studies from our laboratory have described pre- and postnatal recruitment of F4/80+ monocytes into retina (Hume et al. 1983) and brain (Perry et al. 1985) in response to natural death of neurons and axons.

Apart from phagocytosis of dead cells, the functions
of MΦ in the fetus are unknown. Identification and localization of MΦ in tissues at different stages of development is a first step towards defining the ontogeny of the mononuclear phagocyte system and the role of macrophages in development of other tissues. In the present study, we have used the F4/80 mAb to analyse the distribution of MΦ in the fetal and neonatal mouse, with special reference to haemopoietic tissues.

Materials and methods

Animals

Embryos and newborn animals were obtained from the F2 generation of CBA/J×C57BL6/J matings. Females were inspected daily and for the presence of a vaginal plug (designated day 0 of pregnancy).

Immunocytochemistry

Fixation and sectioning

Embryos from day 6 to birth and neonatal animals up to 2 wks after birth were examined. Specimens were collected from at least three animals in a single litter and from at least two different litters for each time point. Conceptuses were freed from their decidua and organs were dissected out when they were readily identifiable. Tissues studied were visceral yolk sac, liver, spleen, bone marrow, thymus, lung, kidney and gut. Whole embryos and dissected organs were extensively washed in phosphate-buffered saline (PBS) before immersion in Bouin’s fixative (10% formaldehyde in 75% picric acid and 5% acetic acid) which gave excellent morphology and preservation of the F4/80 ag. Samples were fixed 4–24 h, depending on the size of the tissue, and stored in 70% ethanol. Femora from neonatal animals were first decalcified in a 13% sodium citrate solution at pH 4.7 overnight before immersion in a 1% sodium tetrahydrochloride (Polysciences, Inc., Northampton) and used as advised. The presence of ag was commercially (Vectastain ABC kit, Vector Laboratories, Peterborough) with a melting point of 57 °C. Sections were cut on a Leitz sledge microtome at 5 μm and floated onto 4-well glass slides.

Ab staining

Sections were stained using the avidin–biotin peroxidase method (Hsu et al. 1981). Wax sections were rehydrated and washed in PBS and endogenous peroxidase activity inhibited by incubation in 0.3% H2O2 in methanol for 30 min. After washing in PBS, sections were treated with a 1:100 dilution of normal rabbit serum for 1 h. The MΦ-specific rat anti-mouse F4/80 mAb (Austyn and Gordon, 1981) was used at saturation and the sections incubated for 90 min. Control sections were left in rabbit serum. Biotinylated second Ab and the avidin–biotin–peroxidase complex (ABC) were obtained commercially (Vectastain ABC kit, Vector Laboratories, Peterborough) and used as advised. The presence of ag was revealed by incubation with 0.5 mg ml–1 diaminobenzidine tetrahydrochloride (Polysciences, Inc., Northampton) and 0.02% H2O2 in 10 mM Imidazole pH 7.4. Sections were counterstained in Mayers haematoxylin for 30 s and mounted in DPX. Representative photographs were taken using a blue filter which intensifies the brown precipitate except when more detail of tissue morphology was required, when a blue–green filter was used. Those sections in which either the primary Ab, second Ab or ABC reagent were omitted were negative, as were sections treated with unrelated mAbs.

Quantitation of F4/80+ cells in tissue sections

Sections stained with F4/80 were used to estimate numbers of MΦ in random fields from 5 μm thick sections of haemopoietic organs. F4/80+ cells were counted in 10–20 fields in sections from at least three different animals.

Results

Initial appearance of F4/80-positive MΦ

Haemopoietic stem cells are found in the visceral yolk sac blood islands between days 7 and 8 of embryonic development, although differentiated nucleated blood cells are not seen until a few days later. F4/80 ag, a marker of mature MΦ, was absent from all 6-, 7- and 8-day-old embryos. Serial sections were examined from at least 10 embryos at each of these stages and the survey also included the extraembryonic membranes (visceral yolk sac, amnion, Reichert's membrane and associated parietal endoderm, the ectoplacental cone and extraembryonic ectoderm). In serial sections of sixteen day 9 conceptuses, F4/80+ cells were found only in a single animal in which a few positive monocytes were seen in the yolk sac blood islands, head region, heart and dorsal aorta. By day 10, the vitelline and embryonic circulation is well established and F4/80+ cells were found consistently in all animals in the yolk sac and the mesenchyme.

MΦ in developing lymphohaemopoietic organs

Visceral yolk sac

The yolk sac consists of an endodermal epithelium and underlying mesoderm within which blood islands and vessels develop. Haemopoiesis within blood islands yields mainly erythroid cells and MΦ. F4/80+ monocytes were infrequent in the yolk sac vessels at all ages, although occasional cells were seen attached to the endothelium. A more abundant population of F4/80+ MΦ was observed extravascularly within yolk sac mesoderm, between the visceral and serosal basement membranes. Cells were first seen on day 10 as rounded monocytes that had presumably recently migrated from the vessels (Fig. 1a). During the next 2 days, their numbers increased greatly and F4/80+ plasma membranes could be seen extensively spread underneath endodermal cells (Fig. 1b). They were often found just beneath endothelial cells and appeared in isolated cases to contribute to the vessel wall (Fig. 1c). Subsequently MΦ numbers declined and by day 14 onwards only a few positive cells and stained membrane fragments were evident (Fig. 1d).

Liver

The liver develops as a result of an interaction between invading mesenchyme of the septum transversum and foregut endoderm. Initiation of haemopoiesis is thought to depend on colonisation of this rudiment by multipotential stem cells from the yolk sac at the 28th somite stage (day 10). Embryos were examined prior to and after this day. Entire day 9 embryos examined serially did not contain any F4/80+ cells in the region of...
Mouse macrophages detected by mAb F4/80

Fig. 1. Distribution of F4/80 + cells in yolk sac. (a) 10 day yolk sac. A few F4/80 + monocytes (arrows) are seen between the endoderm (end) and mesothelium (mes), outside the blood islands. Yolk sac erythropoiesis is restricted to a primitive generation of erythroblasts (Eb) within islands. (b) 12 day yolk sac. Increased numbers of MΦ lie beneath endoderm and around vessels. F4/80 labelling revealed cells with extensive plasma membrane processes (arrows). (c) 13 day yolk sac. MΦ are found within stroma of the yolk sac, beneath endothelium and within the vessel wall (arrows). F4/80 + cells were rare within the vessel lumen, except for occasional monocytes and cells traversing endothelium. Vessels are filled with primitive nucleated Eb and mature non-nucleated forms derived from liver (E). (d) 16 day yolk sac. Towards the end of gestation, the yolk sac forms a thickened basement membrane, MΦ numbers decline and only few F4/80 + cells remain (arrows). Bar, 50 μm.

developing liver. F4/80 + monocyte-like cells and large nucleated basophilic yolk sac-derived erythroblasts (Eb) were observed in the sinuses of day 10 livers (Fig. 2a). By day 11, a large population of well-spread MΦ had invaded the tissue and endogenous erythropoietic activity was visible in extrasinusoidal spaces (Fig. 2b). There was considerable expansion of the MΦ population as haemopoiesis progressed (Fig. 2c). A striking feature of F4/80-stained fetal liver was the association between extensively spread MΦ and developing Eb. Distinct MΦ-Eb clusters were obvious with groups of erythroid cells enveloped by F4/80 + MΦ plasma membrane processes (Fig. 2d). Pyknotic nuclei were frequent within MΦ and mature anucleate erythrocytes were observed in hepatic vessels. After birth, declining haemopoietic activity in liver was associated with a decrease in density of the MΦ population. 3-day newborn liver still contained isolated foci of immature erythroid cells with central MΦ (Fig. 2e). At this time, spindle-shaped F4/80 + Kupffer cells became obvious in the liver sinusoids, but they were not associated with remaining erythrocytoid cells (Fig. 2f). After one week, there were a few haemopoietic clusters which disappeared within the next few days, leaving a staining pattern typical of adult liver.

Spleen

Spleens were first recovered from day 12 embryos as a mesenchymal mass attached to the dorsal wall of developing stomach. Even at this stage, yolk-sac-derived nucleated Eb and F4/80 + monocytes were visible in spaces between mesenchymal cells (Fig. 3a). Over the next few days, increased numbers of F4/80 + cells invaded the tissue where they assumed a more ramified morphology. Beginning on day 15, the spleen was an active site of haemopoiesis and developing red cells were seen clustered around extensively spread F4/80 + MΦ. Compared with haemopoiesis in yolk sac and liver, spleen haemopoiesis was less restricted with immature myeloid cells and megakaryoblasts well represented and mature anucleate erythrocytes and polymorphonuclear leukocytes obvious in sinuses and vessels. MΦ and monocytes were labelled throughout fetal spleen, which contained no defined white pulp at this stage. Little lymphopoiesis occurs in spleen until after birth, when small regions of white pulp become evident (Fig. 3b). The density of F4/80 + cells continued to increase and one week after birth heavily labelled red pulp could be clearly distinguished from F4/80-negative white pulp, corresponding to the staining pattern in the normal adult spleen (Hume et al. 1983).

Thymus

At day 13, the epithelial thymic lobes are surrounded by a thin outer capsule of mesenchyme. Flattened F4/80 + cells were seen to lie beneath and within this compact mesenchyme, but were not observed in the inner epithelial mass (Fig. 3c). The mesenchyme subsequently invades and compartmentalizes the lobes of the thymus. At day 14, F4/80 + MΦ were found in the
were not found within the epithelium of these organs. At this stage, F4/80 + cells were randomly distributed in morphology from monocytes to cells with more extensive processes was found and F4/80+ mitoses were observed. F4/80+ mitotic figures were observed. MΦ numbers increased with growth of the organ and the cells became more stellate in appearance. At birth, cortical and medullary regions can be distinguished. The majority of F4/80+ MΦ were found in cortical regions amidst developing thymocytes. Positive cells were also noted in the medulla, but were less common and less extensively spread than the cortical population. MΦ continued to be a feature of the outer capsule now comprising loose connective tissue.

Bone marrow

Formation of the marrow cavity on day 16 results from infiltration of the cartilaginous rudiment by surrounding mesenchyme which contained haemopoietic precursors and F4/80+ MΦ. This MΦ population expanded rapidly after initiation of haemopoietic activity on day 17 (Fig. 4a). The marrow is actively granulopoietic before birth and all stages of granulocytic differentiation were observed in extravascular stroma between bony trabeculae (Fig. 4b). F4/80+ stromal MΦ were seen amongst these early cells and more flattened MΦ lined bone and the sinusoidal cavities, which often also contained labelled monocytes. After birth the marrow also supports erythropoiesis and distinct strands of haemopoietic tissue containing F4/80+ stromal MΦ, Eb and myeloid cells were evident (Fig. 4c). MΦ numbers were considerably increased and arborising F4/80+ plasma membrane processes could be seen between immature haemopoietic cells (Fig. 4d).

MΦ in non-haemopoietic tissues

At mid-gestation, mouse embryos contain large regions of undifferentiated mesenchyme within which organs develop. F4/80+ MΦ were dispersed within loose connective tissue especially surrounding the neuroectoderm (Fig. 5a). Monocytes were seen within vessels, but most F4/80+ cells were extravascular. They were not associated in clusters with other cells as in haemopoietic tissues and their morphology varied from rounded to stellate, with short stubby plasma membrane processes (Fig. 5b). During mesenchymal differentiation, MΦ were excluded from areas where cell specialization became evident, for example, during formation of somites (Fig. 5c).

Developing organs were examined from day 11 (lung) and day 12 (kidney and gut). The mesenchyme surrounding the epithelial layer becomes compacted and contains yolk-sac-derived Eb in primitive vessels. At this stage, F4/80+ cells were randomly distributed within mesenchyme (e.g. in lung, Fig. 5d). A spectrum in morphology from monocytes to cells with more extensive processes was found and F4/80+ mitoses were noted. The number of labelled cells increased concomitantly with the growth of the organ. MΦ were not obviously associated with other cells although some were phagocytic and contained pyknotic nuclei. MΦ were not found within the epithelium of these organs.

During organogenesis, mesenchyme gradually disap-
developing liver. Cell numbers peaked on day 15 and subsequently declined gradually with cessation of haemopoietic activity. The decrease in liver MΦ labelling density between days 16 and 18 coincided with appearance of increased numbers of MΦ in developing stroma of spleen and bone marrow. The number of MΦ in stroma of these haemopoietic organs continued to increase until by post-natal day 7 (spleen) and day 14 (bone marrow) when their density was similar to that found in the adult.

Discussion

In this study, we have used a highly specific rat mAb,
F4/80, directed against a murine plasma membrane differentiation ag of unknown function, to define the appearance, distribution and cellular associations of MΦ during murine development. We have shown that monocytes and MΦ appear sequentially in yolk sac, liver, spleen and bone marrow during haemopoiesis, and that MΦ are present throughout the interstitium during organogenesis. MΦ participate in non-phagocytic haemopoietic cell interactions as well as in phagocytosis of dying cells and erythrocyte nuclei, and constitute a major haemopoietic cell population during growth and remodelling of fetal tissues.

The F4/80 ag is relatively stable to fixation and its surface expression makes it possible to define cellular
contacts with good preservation of morphologic detail. A wide range of studies has established that F4/80 expression is present only on monocytes and mature MΦ, and that most MΦ populations in adult murine tissues are labelled (Gordon et al. 1988a). In the adult, F4/80 expression is absent on MΦ in T lymphocyte-dependent areas such as white pulp in spleen (Hume et al. 1983b) and is dim on some monocytes and alveolar MΦ. Our studies on fetal tissues confirm that F4/80 is an efficient and sensitive marker to identify MΦ during development, as shown previously in the nervous system (Hume et al. 1983a) and establish that MΦ are...
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Fig. 6. Appearance of \( \text{M}\Phi \) in relation to haemopoiesis during development. The frequency of \( \text{M}\Phi \) in haemopoietic tissues was estimated from F4/80-stained tissue sections. Random fields were selected using a 40× objective and F4/80 + stellate cells not including monocytes counted in 10–20 fields per tissue. Endogenous haemopoietic activity assessed as clusters of Eb or myeloid cells within the tissue was readily distinguishable from circulating cells in sinususes and tissue spaces. Stromal F4/80 + \( \text{M}\Phi \) were associated with haemopoietic cell clusters in fetal liver, spleen and bone marrow, but not in yolk sac. Amongst the earliest haemopoietic cell populations found in the embryo.

F4/80 + cells were first noted in visceral yolk sac on day 10, and increased in numbers rapidly thereafter in liver, spleen and bone marrow in parallel with haemopoietic activity. F4/80 + cells were also widely distributed throughout the rest of the fetus from day 12. Our studies are compatible with earlier findings (reviewed by Metcalf and Moore, 1971) concerning migration and seeding of haemopoietic progenitors to different tissues during development. F4/80 labelling showed that monocytes could differentiate into rounded or stellate sinusoidal and extravascular \( \text{M}\Phi \) in fetal tissues. Since \( \text{M}\Phi \) were found to be widely distributed in tissues after day 10, when a circulation has been established within the fetus, it is possible that monocytes are delivered to peripheral sites from sites of production in haemopoietic organs. Few F4/80 + monocytes were detected within blood vessels suggesting that they entered tissues rapidly. However, extravascular \( \text{M}\Phi \) in the fetus proliferated extensively, unlike in the normal adult animal, since F4/80-labelled mitoses were relatively abundant in non-haemopoietic tissues. The signals involved in monocyte-restricted recruitment to fetal tissues are not known. Few granulocytes are produced \textit{in vivo} until day 16, although committed progenitors from earlier stages can give rise to both \( \text{M}\Phi \) and granulocytic series \textit{in vitro} (Johnson and Burgess, 1978). It is therefore likely that lineage-restricted colony stimulating factors are responsible for the vigorous growth of \( \text{M}\Phi \) in the fetus. It is also not clear whether monocyte recruitment to non-haemopoietic tissues depends on chemotactic signals generated by dying cells, as might be the case in the developing nervous system. The present studies indicate that control of \( \text{M}\Phi \) growth and distribution differ in the fetus and the normal adult.

The sequential appearance of F4/80 + cells in yolk sac, liver, spleen and bone marrow was correlated with striking changes in haemopoietic activity at these sites. Morphologic studies revealed intimate associations and cluster formation of stromal \( \text{M}\Phi \) with erythroblasts in fetal liver, spleen red pulp and bone marrow, but not in yolk sac, where F4/80-labelled cells were not associated with erythroblasts. Kinetic analysis showed that production of erythroid cells preceded that of monocytes and \( \text{M}\Phi \) in yolk sac, but followed the appearance of stellate \( \text{M}\Phi \) in fetal liver and at subsequent sites of haemopoiesis. This observation suggests that interactions with stromal \( \text{M}\Phi \) may be required for erythroid cell differentiation in fetal liver, but not in yolk sac. Stable haemopoietic clusters have been isolated by collagenase digestion of fetal liver at day 14 and Eb binding to \( \text{M}\Phi \) shown to be due to a divalent cation-dependent adhesion receptor (Morris et al. 1988). Fetal liver Eb are known to differentiate further than the more primitive yolk sac Eb, and earlier studies (Cudennec et al. 1981; Labastie et al. 1984) provided evidence that mouse yolk sac haemopoietic precursors depend on extrinsic diffusible factors to induce adult erythropoiesis, but did not identify their source within the fetal microenvironment. \( \text{M}\Phi \) and their products, including erythropoietin (Gruber et al. 1977), may promote erythroid differentiation in liver and subsequently in the spleen and bone marrow. Apart from a possible trophic function (Crocker et al. 1988), stromal \( \text{M}\Phi \) phagocytose erythroid nuclei generated during definitive erythropoiesis. Further studies are needed to establish the nature and functional significance of the interactions between stromal \( \text{M}\Phi \) and developing erythroid cells.

Myeloid cells also cluster with stromal \( \text{M}\Phi \) at later stages of haemopoiesis in spleen and bone marrow, and a second haemopoietic cell interaction receptor, sia-loadhesin (Crocker and Gordon, 1989) appears on stromal \( \text{M}\Phi \) in liver, spleen and bone marrow after d18.


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