Macrophage recruitment during limb development and wound healing in the embryonic and foetal mouse

James Hopkinson-Woolley¹, Derralynn Hughes², Siamon Gordon² and Paul Martin³*,

¹Department of Human Anatomy, Oxford University, South Parks Road, Oxford OX1 3QX, UK
²Sir William Dunn School of Pathology, Oxford University, South Parks Road, Oxford OX1 3RE, UK
³Department of Anatomy and Developmental Biology, and Department of Plastic Surgery, University College London, Gower Street, London WC1E 6BT, UK

*Author for correspondence

SUMMARY

Macrophages play a pivotal role in the adult inflammatory response towounding. They are directly responsible for cellular débridement and, by providing a source of growth factors and cytokines, they recruit other inflammatory and fibroblastic cells and influence cell proliferation and tissue remodelling. In this paper we investigate the role of macrophages in clearing areas of programmed cell death in the developing embryo and also their role in embryonic and foetal wound healing. Immunocytochemistry using the monocyte/macrophage-specific monoclonal antibody, F4/80, reveals a close association between areas of programmed cell death in the remodelling interdigital regions of the mouse footplate and of F4/80-positive cells, suggesting that monocyte-derived macrophages, and not locally recruited fibroblastic cells, as previously believed, are responsible for phagocytosing and clearing areas of interdigital apoptosis. Our studies of wound healing reveal that macrophages are not recruited to, and therefore cannot be playing an active role in the healing of, excisional wounds made in the mouse embryo at any stage up until E14.5. Beyond this transition stage we see a significant recruitment of macrophages within 12 hours of wounding. We find that macrophages can be attracted to wounds in earlier embryos if the wound results in significant cell death such as after burning.

Key words: macrophage, F4/80, apoptosis, necrosis, wound-healing, embryo, limb-bud

INTRODUCTION

Embryos and early-stage foetuses are extremely efficient at tissue remodelling both after periods of naturally occurring programmed cell-death (Hinchliffe, 1981; Clarke, 1990) and also after artificially induced tissue damage caused by wounding (Radice, 1980; Martin and Lewis, 1992; Martin and Nobes, 1992; Whitby and Ferguson, 1991a,b; Lorenz et al., 1992). In this paper we investigate the role played during these two processes in the embryo by the macrophage, a cell type known to have a central role in the processes of inflammation and tissue remodelling in adult tissues.

In the adult, macrophages appear to play a significant role in almost all inflammatory responses. For example, they invade sites of incisional or excisional wounds within about 6 hours (Van Furth et al., 1985; reviewed by Riches, 1988) and, once there, they phagocyte cellular debris and release a plethora of growth factors and cytokines, many of which are probably important in the wound repair process (reviewed by Martin et al., 1992). It has been shown in adult guinea pigs that if the macrophage influx is blocked by administration of anti-macrophage serum and steroids, then wound healing is severely hindered (Leibovich and Ross, 1975).

Previous studies in the mouse embryo have shown that the macrophage as a cell lineage first appears on about the 10th day of gestation in the yolk sac and liver and then in the spleen and surrounding mesenchymal tissues (De Felici et al., 1986; Takahashi et al., 1989; Morris et al., 1991). Numbers of macrophages increase in all tissues from that stage on, with the most significant increases apparent in the liver, spleen and bone marrow as haemopoiesis begins in these tissues (Morris et al., 1991). Similar studies in the chick embryo show yolk-sac-derived macrophages to have similar tissue distribution in the avian embryo also (Cuadros et al., 1992). Given that large numbers of this specialised cell type are present throughout the tissues of the embryo from about mid-gestation onwards, what roles might they play in normal development and in inflammatory responses in the embryo?

We have used the macrophage-specific monoclonal antibody F4/80 (Austyn and Gordon, 1981) to investigate, first of all, the role played by macrophages in phagocytic clearing of naturally occurring programmed cell death in the remodelling interdigital zones of the developing mouse footplate. Previous studies in avian and mammalian embryos have documented the presence of phagocytic cells in the interdigital tissue by histological methods but the majority of such studies, whilst calling these cells macrophages, have reported them to be non-specialist cells derived from fibroblastic cells in the vicinity of the
interdigital zone (Ballard and Holt, 1968; reviewed by Hinchcliffe and Johnson, 1980; Garcia-Martinez et al., 1993). We show here that these phagocytic cells are in fact monocyte-derived macrophages and that they are first recruited at, or shortly after, the onset of cell death in the mesenchyme of the interdigital space.

Since the macrophage appears to play such a pivotal role in adult tissue repair (Leibovich and Ross, 1975), it is of interest to discover if it plays a similarly important role in embryonic and foetal healing. Previous studies have suggested that only a weak inflammatory response is triggered in the foetus after wounding. Whitty and Ferguson (1991a), for example, report no detectable endogenous IgG or IgM immunoglobulins at healing incisional lip wounds in E14 foetal mice and, using various histochemical and morphological criteria, a number of studies in rabbit and sheep report no significant inflammatory cell recruitment to wounds until late stages of gestation (Burrington, 1971; Adzick et al., 1985). Using the monocyte/macrophage-specific antibody F4/80, we have made a comprehensive study of macrophage recruitment to wounds made at various stages of development and our studies reveal that a transition occurs at about E14.5. Wounds made before this stage recruit very few, if any, macrophages as they are closing, whereas in lesions made later than E14.5 we see a substantive influx of macrophages to the wound site within 12 hours of wounding.

**MATERIALS AND METHODS**

For these studies we used an outbred strain of Albino mouse, strain PO (Pathology, Oxford). Gestational age was calculated on the assumption that conception had occurred at midnight preceding the morning on which a vaginal plug was first observed. Since embryos within a litter vary considerably with regard to actual developmental stage, embryos were individually staged according to Theliller (1989) and Martin (1990), primarily according to the degree of development of the fore- and hindlimb buds. Sibling embryos of almost identical developmental stage were processed in parallel for resin histology and various histochemical and morphological criteria, a number of studies in rabbit and sheep report no significant inflammatory cell recruitment to wounds until late stages of gestation (Burrington, 1971; Adzick et al., 1985). Using the monocyte/macrophage-specific antibody F4/80, we have made a comprehensive study of macrophage recruitment to wounds made at various stages of development and our studies reveal that a transition occurs at about E14.5. Wounds made before this stage recruit very few, if any, macrophages as they are closing, whereas in lesions made later than E14.5 we see a substantive influx of macrophages to the wound site within 12 hours of wounding.

**Embryo wounding in culture**

Embryos were dissected from the uterus into sterile Tyrode’s saline at E11.5, E12.5 and E13.5. To prepare embryos for culture the decidua were first trimmed away and the yolk sac cut adjacent to the placenta. Embryos were individually staged according to Theliller (1989) and Martin (1990), primarily according to the degree of development of the fore- and hindlimb buds. Sibling embryos of almost identical developmental stage were processed in parallel for resin histology and various histochemical and morphological criteria, a number of studies in rabbit and sheep report no significant inflammatory cell recruitment to wounds until late stages of gestation (Burrington, 1971; Adzick et al., 1985). Using the monocyte/macrophage-specific antibody F4/80, we have made a comprehensive study of macrophage recruitment to wounds made at various stages of development and our studies reveal that a transition occurs at about E14.5. Wounds made before this stage recruit very few, if any, macrophages as they are closing, whereas in lesions made later than E14.5 we see a substantive influx of macrophages to the wound site within 12 hours of wounding.

**Open uterus embryo/foetal wounding**

Manipulation of embryos in vivo was achieved through open uterus surgery as slightly modified from Muneoka et al. (1990). Pregnant mice carrying embryos of gestational age E13.5 and E14.5 were anaesthetised with a 1:1:2 mix of Hypnorm:Hypnovel (Roche):Ringer-lactate solution at 0.1 ml per 10 g body weight. The mother’s abdomen was cleaned using a depilatory cream (Immac, Reckitt) and ethanol, and a midline incision was made through the skin and abdominal wall. The abdominal cavity was filled with warmed Ringer-lactate solution, the uterus opened along its anterplacental border and all but four foetuses - two in each horn of the uterus - were removed by rolling the placenta off the uterus with a sterile cotton bud and discarded. Of the four remaining foetuses only one in each horn was operated on. For each of these foetuses a small hole was made in the yolk sac adjacent to the limb and an excisional wound, of similar size to the amputation wound previously made to E11.5 embryos, was made to the footplate of the foetal limb. The hole in the yolk sac was then closed with a 10/0 monofilament suture (Ethicon) and when both foetuses had been operated on, the mother’s abdominal cavity was rinsed out with fresh Ringer-lactate solution and her abdominal wall and skin wound were closed with a 6/0 monofilament suture. Foetuses were harvested 12, 24 and 48 hours after wounding. A total of 8 E13.5 and 12 E14.5 wounded foetuses were studied, and all of them survived surgery until we harvested them.

**Scanning electron microscopy and resin histology**

Embryos for scanning electron microscopy (SEM) or resin histology were rinsed in PBS, fixed overnight in ice-cold half-strength Karnovsky fixative (Karnovsky, 1965), rinsed in buffer and post-fixed in 1% osmium tetroxide before being dehydrated through a graded ethanol series. Specimens for SEM were then critical-point-dried in CO2 and sputter-coated with gold before viewing with a Philips 515 microscope. Specimens for resin histology were embedded in Araldite and sections of 1-5 μm cut and stained with Toluidine Blue.

**F4/80 immunocytochemistry**

In preparation for immunocytochemistry, embryos were fixed in 4% paraformaldehyde in PBS for 1-2 hours and washed with PBS before being transferred to ethanol series. Embryos were then rehydrated and rinsed with fresh Ringer-lactate solution and her abdominal wall and skin wound were closed with a 6/0 monofilament suture. Foetuses were harvested 12, 24 and 48 hours after wounding. A total of 8 E13.5 and 12 E14.5 wounded foetuses were studied, and all of them survived surgery until we harvested them.

**Fig. 1.** Sections through the developing footplate to show the correlation of morphological cell death with immunocytochemical staining for macrophages at E12.5 (A,B,C); E13.5 (D,E,F,G); and E14.5 (H,J,J). (A) Low-magnification Araldite section of the E12.5 footplate. (B) High-power detail of A showing the anterior margin of the footplate where there are numerous pyknotic nuclei. Most are in clusters as though already engulfed by phagocytic cells (arrows). (C) F4/80 immunocytochemistry of a region equivalent to that shown in B reveals a collection of macrophages in this early site of programmed cell death (arrows). (D) Low-magnification Araldite section of an E13.5 footplate. Programmed cell death is occurring in the interdigital regions indicated with arrows. (E) High-magnification detail of the interdigital region indicated with an asterisk in F to show macrophage recruitment into the zone of the interdigital cell death. (G) High-magnification detail of the region indicated with an asterisk in E to show macrophage recruitment into the zone of the interdigital cell death. (H) Low-magnification Araldite section of an E14.5 footplate reveals the digits are almost formed. (I) Higher-power detail of G corresponding to the region indicated with an asterisk in F to show macrophage clusters as though already engulfed by phagocytic cells (arrows). (J) F4/80 immunocytochemical staining of a similarly staged footplate to that shown in I showing the collections of macrophages lying along the lateral margins of each digit. Bars: A,D,F,H,J, 500 μm; B,C,E,G,I, 100 μm.
overnight in Bouin’s fixative and thoroughly rinsed in 70% ethanol prior to further dehydration and embedding in wax. Sections (5 μm) were cut and mounted on albumin-coated slides. To reveal macrophages, rehydrated sections were first rinsed in phosphate buffered saline (PBS) and endogenous peroxidase activity was then inactivated by a 30 minute immersion in 0.3% H₂O₂ in methanol. After further PBS rinses the sections were treated for 30 minutes with rabbit serum (1:100) to block non-specific antibody binding. The
macrophage-specific rat anti-mouse F4/80 antibody (1:20) (Austyn and Gordon, 1981; Morris et al., 1991; Gordon et al., 1992) was applied to sections for 90 minutes, after which the sections were again thoroughly rinsed in PBS. Bound antibody was revealed using a biotinylated anti-rat IgG (made in rabbit and mouse adsorbed; Vector Labs) and the avidin-biotin-peroxidase complex (Vectastain ABC kit, Vector Labs). Peroxidase activity was detected by incubation in 0.5 mg/ml diaminobenzidine tetrahydrochloride (DAB) in 10 mM imidazole, at pH 7.4, with an equal volume of 0.02% H2O2. These sections were then washed in tap water and lightly counter-stained with Mayer’s Haemalum. At least three specimens for each time-point after wounding of each stage of embryo and foetus were processed for F4/80 immunocytochemistry and all sections through each wound region were inspected for macrophage recruitment.

Those sections in which either primary antibody, second antibody or ABC reagent were omitted were negative.

RESULTS

Previous studies have shown that macrophages are present in the haemopoietic tissues of the mouse embryo from about E10 and are present in low numbers in the connective tissues of the embryo proper from about E10.5 onwards (Morris et al., 1991). In our studies of macrophage involvement in normal programmed cell death and in embryonic wound healing, we have focussed on stages subsequent to this.

**Interdigital cell death and macrophages**

Transformation of the hindlimb footplate into a foot with separate digits occurs between about E12.5 and E14.5 in the mouse embryo (Theiler, 1989; Martin, 1990). Resin sections of the E12.5 footplate show clusters of pyknotic cells at the extreme anterior and posterior margins (Fig. 1A,B). At this stage there is very little, if any, cell death in the mesenchyme of the interdigital zones. F4/80 staining reveals that macrophage distribution correlates precisely with the distribution of programmed cell death - aggregations of F4/80 cells (many of them full of phagocytosed cellular debris) are present at the anterior and posterior limb margins (Fig. 1C), and no macrophages are seen in the central interdigital regions of the footplate.

At E13.5, resin sections reveal that large numbers of pyknotic cells are still present anterior to digit 1 and posterior to digit 5, but now there are also apoptotic cells in the first and fourth interdigit spaces and to a lesser extent in the second and third interdigit spaces (Fig. 1D,E). Again our F4/80 study shows that the distribution of macrophages correlates well with the localisation of apoptotic cells, with macrophages now also present in all of the interdigital spaces (Fig. 1F,G).

By E14.5 interdigital cell death has clearly remodelled the footplate so that the toes are now separated. However, there is still more cell death occurring at the anterior and posterior margins of each toe (Fig. 1H,I). Wherever clusters of pyknotic cells are found we also find collections of macrophages in similar sections from sibling embryos processed for F4/80 immunocytochemistry (Fig. 1J). Many of these macrophages are very heavily laden with phagocytosed cellular material.

**Wound healing at various stages of embryonic development**

We wounded the hindlimb of mouse embryos at stages ranging from E11.5, when the limb is just a bud, up to E14.5, when limb patterning is almost complete. So that the wounds inflicted at different stages could reasonably be compared, we tried to keep their dimensions similar; to this end, at E11.5 we excised the whole limb-bud from the flank (Fig. 2A); at E12.5 the whole footplate was amputated at the level of the presumptive ankle (Fig. 2B); at E13.5 a wedge of tissue corre-
sponding to the middle three digits was cut away (Fig. 2C); and at 14.5, one toe (usually digit 5) was amputated at its base (Fig. 2D). Wounds at each of these stages resulted in cutting of several large vessels and many smaller vessels, but in all cases bleeding ceased within about 3 minutes. Resulting wound areas were approximately 0.35 mm² (E11.5), 0.4 mm² (E12.5), 0.25 mm² (E13.5) and 0.15 mm² (E14.5).

Embryos wounded at E11.5 and E12.5, and some of those wounded at E13.5, were cultured in roller bottles as described in Materials and Methods. The remaining E13.5 and all E14.5 embryos were operated on by open-uterus surgery and remained in the mother’s peritoneal cavity during the period of wound healing. E11.5 wounds healed rapidly as previously reported (McCluskey et al., 1993; Martin et al., 1993); almost all the wounds that we examined were fully closed after only 24 hours of culture. Wounds at later stages took progressively longer to heal: about 36 hours to cover the ankle amputation of an E12.5 wound and up to 2 days to heal a toe amputation in the E14.5 embryos (data not shown).

**Macrophages are not recruited to wounds healing in E11.5, E12.5 or E13.5 embryos until after the wound has closed**

Embryos wounded at E11.5 were fixed and processed for F4/80 immunocytochemistry after 12, 18 and 24 hours of culture; by 24 hours all wounds had closed as reported above and previously (McCluskey et al., 1993; Martin et al., 1993). At 12 and 18 hours after wounding, whilst occasional macrophages were seen in and around the wound site, no significant recruitment of macrophages to the wound itself was seen (Fig. 3A,B). Numbers of macrophages seemed comparable to or less than those seen at the equivalent region at the base of the limb on the contralateral unwounded side of the embryo or in other regions of the embryo. No significant accumulation of macrophages was observed until 24 hours of culture when in two of the three specimens examined we found a significant aggregation of macrophages beneath where the epidermal wound edges had just met (Fig. 3C). This collection of macrophages correlated with where wound debris - shunted ahead of the epidermal fronts as they swept in over exposed connective-tissue - had been deposited as the epidermal wound edges met at the focal point of closure.

Embryos wounded at E12.5 (Fig. 3D,E) and E13.5 (both those specimens cultured in vitro and those maintained in open-uterus conditions) (Fig. 3F,G) appeared, like their E11.5 counterparts, not to recruit macrophages whilst the wound was still closing at 12 or 24 hours. Wounds in E13.5 foetuses traversed zones of interdigital cell death with their accompanying large populations of macrophages, but even with such a nearby source of macrophages almost none were recruited to the wound surface (Fig. 3G).

**Wounded E14.5 foetuses reveal significant and rapid macrophage recruitment within 12 hours of wounding**

Foetuses wounded at E14.5 and grown in open-uterus conditions, in contrast to their younger counterparts, revealed a significant macrophage recruitment beginning within 12 hours of wounding. Specimens were examined at 12 hours (Fig. 3H,I), 24 hours (Fig. 3J,K) and 48 hours, and at all of these time-points there was a clear collection of macrophages littering the wound surface. Many of the latter were swollen with phagocytosed material. In some specimens the macrophages appeared to be streaming to the wound from an adjacent interdigital space (Fig. 3K), whilst in others the wound macrophages appeared more isolated from adjacent populations of macrophages in the limb (Fig. 3I).

**Cell death at the wound site caused by a burn lesion is associated with macrophage recruitment in E11.5 embryos**

In order to test whether the lack of a macrophage response to wounding a young embryo was due to the absence of a sufficient chemotactic cue from the excisional wound or to an incapacity of embryonic macrophages to respond to such a cue, we used a localised burn to create lesions with increased amounts of cell death in the limb bud at E11.5. Embryos wounded in such a fashion were harvested after 12 hours. Resin and wax histology revealed that the burn wound caused blistering and extensive local cell necrosis, which consistently extended to a depth of between 150 and 200 μm beneath the surface ectoderm (Fig. 4A,B). F4/80 immunocytochemistry revealed a significant macrophage recruitment to these wound sites (Fig. 4C,D) at a developmental age when an excisional lesion would have remained macrophage-free.

**DISCUSSION**

In this paper we have used the monoclonal antibody F4/80 to investigate macrophage recruitment to regions of programmed cell death in the interdigital spaces of the developing mouse hindlimb and to the sites of healing wounds in the hindlimb inflicted at various developmental ages. We find a clear spatial and temporal association of F4/80-positive macrophages with regions of programmed cell death in the limb interdigital spaces, from the onset of interdigital cell death at E12.5 until E14.5 when the footplate had almost fully remodeled. A number of previous studies have suggested that monocyte-derived macrophages play several important phagocytic roles during embryogenesis. Their presence in the urogenital ridges suggests a role in clearing programmed cell death after degeneration of the Mullerian duct in male and the Wolffian duct in female embryos, as well as the mesonephros in embryos of both sexes (De Felici et al., 1986). Later in development it has been shown that there is recruitment of F4/80 monocytes into the retina (Hume et al., 1983) and brain (Perry et al., 1985) in response to programmed death of neuronal cells. A recent paper by Lang and Bishop (1993) suggests that macrophages may even be responsible for initiating programmed cell death in some tissues of the developing eye. Until the present study, however, it has generally been believed that apoptotic cells in the remodelling interdigital spaces are engulfed and cleared by neighbouring limb mesenchymal cells (Ballard and Holt, 1968; reviewed by Hinchliffe and Johnson, 1980; Garcia-Martinez et al., 1993). Our study strongly implies that this is not the case; rather we show that there is a significant presence of F4/80 positive cells at all interdigital locations where there is cell death, and many of these monocyte-derived macrophages are laden with numerous intracellular pyknotic nuclei, suggesting that they are actively phagocytosing dying cells. Since our study did not reveal a stage or location in the footplate where
Fig. 3. F4/80 immunocytochemical staining to reveal macrophage response at various time-points after wounding the E11.5 (A,B,C); E12.5 (D,E); E13.5 (F,G) and E14.5 (H,I,J,K) hind-limb. (A) Low-magnification transverse section through an E11.5 wound 12 hours after wounding. (B) Detail of A to show very low numbers of macrophages (brown staining) at the wound site. (C) Section through an E11.5 wound after 24 hours. The wound is now closed and beneath the focal point of epithelial closure (arrow) is a collection of macrophages. (D) A section through an E12.5 healing wound 12 hours after wounding. (E) High-power detail of D showing very few macrophages at the wound site. (F) A section through an E13.5 healing wound 12 hours after wounding. (G) High-power detail of F showing only small numbers of macrophages at the wound surface. (H) A section through an E14.5 footplate 12 hours after amputation of the presumptive big toe. (I) High-power detail of H showing a significant influx of macrophages that have accumulated on the wound surface. (J) A section through another E14.5 footplate but this time the wound has been healing for 24 hours. (K) Detail of J to show that again there is a significant macrophage presence at the wound surface. In all sections arrows mark the epidermal wound edges. Bars: A,D,F,H,J, 500 μm; B,C,E,G,I,K, 100 μm.
apoptotic cells were present but where F4/80 cells had not yet appeared, it would seem that macrophage recruitment is very rapid. Indeed, our study is not inconsistent with the possibility that macrophages might play some role in triggering apoptosis in the interdigital cells as appears to be the case for programmed cell death in the developing eye (Lang and Bishop, 1993). Whilst the capacity to recognise and phagocytose apoptotic as well as necrotic cells is not unique to embryonic macrophages (Fadok et al., 1992), it will be interesting to determine what signals from the interdigital cells lead to such a rapid response by macrophages, and whether the responding macrophages are derived from those already present in the limb mesenchyme or whether there is further recruitment from the pool of circulating blood monocytes.

With regard to macrophage involvement in embryonic and foetal healing, our most important finding is the clear demonstration that macrophages are not recruited to healing wounds in the young (limb bud stage) embryo during the period of wound closure. Only after the wound has closed and a collection of cell debris, including small numbers of necrotic cells, has been deposited at the focal point of closure do we see macrophages infiltrate the wound site. Embryos are clearly quite capable of rapidly and efficiently healing a wound in the absence of macrophages. Leibovich and Ross (1975) revealed that macrophages play an essential role in adult tissue repair and so our data show a clear and dramatic difference in the machinery of embryonic versus adult repair. Megakaryocytes - the progenitor cells of platelets - do not begin to differentiate in the embryonic liver until E13 in the mouse embryo (Rugh, 1990), so both macrophages and platelets, the two major sources of growth factors in the adult wound, are absent from the embryonic wound site. Recent studies of ours show that at least one growth factor, transforming growth factor β1 (TGFβ1), is rapidly upregulated by the epidermal and mesenchymal cells at the edge of the wound and may be involved in signalling contraction of the wound connective tissues (Martin et al., 1993). However, whilst growth factors are present at the embryonic wound site and thus available to signal the tissue movements of wound closure, even at later foetal stages they remain at reduced levels when compared to the growth factor profile seen at an adult wound (Whitby and Ferguson, 1991b).

To test whether embryonic macrophages are incapable of mounting an inflammatory response to a wound, either because at this stage they are still blind to chemotactic signals or simply because there are too few of them to respond, we inflicted localised burn wounds on the E11.5 limb bud. These wounds, which caused a localised focus of cell necrosis, resulted in a significant recruitment of macrophages to the wound site, suggesting that embryonic macrophages are capable of mounting...
an inflammatory response as long as there is sufficient cell-death stimulus. A previous study of ours describes the sparsity of cell death seen at a clean excisional wound site in the E11.5 mouse embryo (McCluskey et al., 1993) and this may explain why macrophages are seemingly oblivious to such a wound until it has closed and the epidermal fronts have concentrated the little cell debris all to one point.

It is interesting to note that embryonic macrophages appear to be recruited both to regions of apoptotic cell death, as in the interdigital spaces, and to collections of necrosis, as in the burn wound. What might the signals be that lead to these recruitments? Whilst it seems likely that macrophages could be attracted to regions of cell death by medium-range chemotactic signals, it is also possible that recruitment is driven by short-range or cell-cell contact-mediated signals and is thus dependent on macrophages constantly patrolling tissues and accumulating wherever they find such signals. Unfortunately, whilst the former seems most likely for macrophage recruitment to wounds and the latter a likely mechanism for recruitment to sites of programmed cell death, our current studies do not allow us to differentiate between the two.

If macrophages are so important for post-natal wound repair, at what stage in foetal development do they begin to respond to an excisional wound by mounting an inflammatory response? Since later stage embryos and foetuses cannot yet be cultured in vitro, we have investigated this question, of necessity, by open-uterus surgery on foetuses. We have found that even though healing occurs efficiently, there is no significant macrophage recruitment until E14.5, at which age we began to see consistently large numbers of macrophages at the wound site within 12 hours after wounding. Possibly, more extensive cell death at the sites of wounds post-E14.5 might explain why these wounds lead to a macrophage recruitment whilst earlier wounds do not. To be sure that the apparent transition from macrophage absence to macrophage recruitment to a wound was not due simply to the fact that early embryos were cultured in roller bottles whilst later foetuses were wounded in situ, we have wounded some intermediate stage - E13.5 - embryos and then cultured them, whilst others were wounded and maintained by open-uterus surgery; both gave the same result of no macrophage recruitment during the observed healing period. Numerous authors report the rapid and efficient wound-healing capacity of the embryo and early foetus, and speculate as to what might be the key differences that lead to scarless healing in the embryo but to less-efficient healing with scars in the late-stage foetus and beyond (for reviews see Adzick and Longaker, 1991). The transition stage after which we see a macrophage recruitment to an excisional wound site is coincident with the stage in mouse foetal development beyond which a large excisional wound results in a defect or scar (Hopkinson-Woolley and Martin, unpublished data), suggesting the possibility that the presence of macrophages at a wound site, whilst apparently essential for efficient tissue repair in the adult, may also be responsible for scar formation. Modulation of growth factor levels at adult wound sites seems able to inhibit scar tissue formation (Shah et al., 1992); our results suggest that modulating macrophage numbers at the wound site may prove to be of similar therapeutic benefit.

In summary, our data reveal that embryonic macrophages are recruited both to sites of programmed cell death (apoptosis), such as during remodelling of the interdigital spaces of the limb, and to sites of necrotic cell death, as in the burn wounds or at the focal point of closure of excisional wounds. However, macrophages are not normally recruited to, and therefore can not be essential for the healing of, excisional wounds made in the mouse foetus earlier than E14.5.

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