OSTEOGENESIS AFTER BONE AND BONE MARROW TRANSPLANTATION

Studies of Cellular Behaviour Using Combined Myelo-osseous Grafts in the Subscorbutic Guinea Pig

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In order to study factors influencing osteogenesis after bone and bone marrow transplantation, we have caused guinea pigs to become scorbutic, and looked at the cell morphology at sites of bone formation. We had previously studied normal guinea pigs and found that autologous marrow in intermuscular implants was associated with bone production by the ninth day, regardless of the type of stored allogeneic bone transplanted with it. In subscorbutic guinea pigs, using identical implants, bone did not appear within the first 13 days, and the cell population around the implants was different. These experiments support the dominant role of bone marrow cells in osteogenesis and cast further doubt on the primary role of devitalised bone as an inducer of bone formation. Interference with cell function by deprivation of a single essential molecule, Vitamin C, produces great change in the ability of cells to synthesise bone, or pre-osseous matrix.

Key words: bone; bone cells; bone marrow cells; bone transplantation; scurvy; Vitamin C

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Bone grafts have been a clinical tool of the orthopaedic surgeon for many years. The current favoured material is fresh autologous cancellous iliac bone. There are numerous clinical reasons for seeking alternatives to this, and our studies have been directed at a better understanding and control of factors influencing osteogenesis. The biological principle of induced osteogenesis offers a therapeutic approach that may find wide use in orthopaedic surgery, and is perhaps different from osteoconductive bone healing (Glowacki et al. 1981).

In a previous paper (Cummine & Nade 1977), we reported our findings after bone and bone marrow grafting in guinea pigs, placing emphasis on factors which contribute to osteogenesis. We concluded that an autografted bone marrow cell population was osteogenic, and new bone formed in sites of transplantation, appearing after the ninth day. The presence of allogeneic bone in the form of a combined myelo-osseous graft, together with autologous marrow cells, had an influence on cell populations found only insofar as non-decalcified bone stimulated the presence of giant cells (Nade 1977).

Decalcification of the allogeneic bone prevented this, but the type of acid used for decalcification had an effect on the amount of newly-formed bone that appeared (Nade & Burwell 1977). We suggested that the physical and chemical nature of the environment of bone marrow cells plays a part in determining their fate after
autologous transplantation. One way of influencing a cell’s synthetic activity is to selectively alter its nutrition.

Vitamin C deprivation affects bone growth and collagen synthesis (Murray & Kodicek 1949a, b, c), as is known from the manifestations of scurvy. We chose to use the guinea pig in the experiments reported herein because that animal, like man, can be made scorbritic.

In this paper we detail the cellular events during the first 13 days following bone and bone marrow transplantation in the subscorbatic guinea pig. Compared with the normal animal (Cummine & Nade 1977), a different cell population was seen, and bone formation did not occur. Furthermore, electron microscopy of the site where bone formation was seen in normal animals showed an abnormal extra-cellular matrix in that region.

Previous studies of bone formation in scorbatic animals (Bonucci 1966, Thyberg et al. 1971) have been directed at the growth plate and adjacent metaphysis of young animals. The ultrastructure of induced bone formation in a muscular site has been studied only by Nilsen (1977, 1980a, b) using implants of allogeneic decalcified dentine.

Rather than examine the presumed changes in osteoblast morphology following withdrawal of ascorbic acid from the diet, as studies about the growth plate have done, out approach has been to use a reproducible model of ectopic bone as the site for study, and to record changes from the normal guinea pig as a means of better understanding osteogenesis.

MATERIALS AND METHODS

The experimental design was similar to that used previously (Cummine & Nade 1977), except that the guinea pigs were made subscorbetic. Their diet consisted of pellets which contained crude protein 20 per cent, crude fat 4.5 per cent, fibre 7.5 per cent, salt 2 per cent, and added Vitamins A, B1, B2, D3, E and nicotinic acid. There was no added Vitamin C, and green vegetable matter was excluded until the fourteenth day after this diet commenced. From that time, green vegetable food was provided to permit animal survival for longer periods. The animals commenced the scorbutogenic diet 7 days before they received implants. The implants used were:

(a) Allogeneic bone alone, both cortical calcified and decalcified, and cancellous calcified and decalcified;
(b) Identical bone, combined with autologous bone marrow just before implantation; and
(c) Autologous bone marrow alone.

The allogeneic bone was taken from non-scorbutic normal guinea pigs. Implant duration periods were 1, 3, 5, 7, 9, 11 and 13 days. There were three animals for each time period, after which the animals were killed.

<table>
<thead>
<tr>
<th>No. of days animal on diet at time of implant insertion</th>
<th>No. of days after diet commenced animal was killed</th>
<th>No. of days after diet was commenced that 'greens' were introduced</th>
<th>No. of days animal was on subscorbatic diet</th>
<th>No. of days implant retained</th>
<th>Principal histological features from 3 animals each period</th>
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<tr>
<td>7</td>
<td>8</td>
<td>–</td>
<td>8</td>
<td>1</td>
<td>Haemorrhage prominent</td>
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<td>7</td>
<td>14</td>
<td>–</td>
<td>14</td>
<td>7</td>
<td>Implanted marrow no longer detected</td>
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<tr>
<td>7</td>
<td>16</td>
<td>14</td>
<td>14</td>
<td>9</td>
<td>Giant cells present in non-decalcified implants</td>
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<td>7</td>
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for implant retrieval. Thus the animals were deprived of Vitamin C for a minimum of 8 days, and a maximum of 14. Twenty-one animals were used, each containing nine different implants in inter-muscular pouches in the anterior abdominal wall (see Table 1).

Standard methods were used for tissue preparation for microscopy.

RESULTS

Histological findings in the subscorbutic animals were:

(i) No newly-formed bone was seen in any section;
(ii) The ovoid cells of uncertain nature, seen at implant sites in normal animals, were rarely seen; and
(iii) Polymorphonuclear leucocytes were prominent at all time intervals (Figure 1).

Examination by electron microscopy of the junctional zone of transplanted marrow and allogeneic bone, the site where new bone usually first appeared in the non-scorbutic guinea pigs, revealed:

(i) An abnormal extracellular matrix consisting of a very loosely-woven network of thin fibres (Figure 2)

(ii) Cells containing granules, which could be ferritin, adjacent to that matrix;
(iii) No cells which could be called osteoblasts;
(iv) No 'osteoid matrix'; and
(v) No matrix vesicles, known to be associated with bone formation.

Even in those animals in whom 'greens' had been re-instituted in the diet, there was no restoration of the normal pattern of bone formation.

These results were different from normal guinea pigs (Cummine & Nade 1977), in which, by the ninth day, new bone was seen in associa-
tion with autologous marrow and allograft bone (Figure 3). In addition, the ovoid cells, which were seen in greatest numbers in association with those implants containing autologous marrow in the control series, were rarely seen in the subscorbutic animal. Polymorphonuclear leucocytes were not prominent.

The only distinct difference between the various implants, regardless of duration of implantation, was the presence of giant cells around the non-decalcified allograft bone.

Haemorrhage resulting from the operative procedure was prominent at all time periods, especially during the first 3 days. Implanted marrow cells could not be recognised by the seventh day. The tendency to form a fibrous capsule was evident by the ninth day, with circumferential orientation of fibroblasts and collagen deposition. However, this fibrous connective tissue did bear the features described under 'scurvy marrow' in the discussion. In other respects, the time sequence of cellular events was similar to that in normal guinea pigs, indicating that not all cellular dynamics were affected by Vitamin C deprivation.

We have used the technique of bone and bone marrow transplantation to extra-osseous sites for a considerable period (Nade 1979) and are familiar with the events which occur at the junction of decalcified allogeneic implanted bone, and newly-formed bone on or adjacent to its surface. As well as conventional histology we have studied the ultrastructure of this region and have found characteristic features in normal animals at this interface (Figure 4).

A monolayer of cells morphologically similar to osteoblasts formed adjacent to the allogeneic decalcified bone. These cells were ovoid or spherical in shape and contained a single nucleus, eccentrically placed. The cytoplasm contained extensive granular endoplasmic reticulum with dilated cisternae. Golgi complexes were present in the paranuclear space. Several layers of closely-packed mononuclear spindle-shaped cells were present adjacent to the osteoblast cells. The extracellular osteoid matrix consisted of randomly-oriented collagen fibres displaying typical crossbanding. In contrast the specimens from scorbutic animals (Figure 5) did not have osteoblast-like cells or the spindle-shaped cells. Granule-containing cells were prevalent in all the specimens examined. These cells may be granular leukocytes or phagocytic cells containing ferritin granules as suggested by Bonucci (1966). The extracellular matrix consisted of a fairly dense network of fine fibres. No obvious collagenous crossbanding was observed.

**DISCUSSION**

The radiological findings in human scurvy are well documented (Park et al. 1935), but his-
osteological studies have mainly been directed at secondary centres of ossification and the enchondral growth plate (Ham & Elliot 1938). Endosteal bone and its contained marrow have rarely been studied — reports (Park et al. 1935) appear to provide similar findings to scorbutic guinea pigs (Murray & Kodicek 1949c). These animals, like man, are unable to synthesise Vitamin C, and were studied in the totally-deprived scorbutic state by Holst & Frolich (1907), and Walbach & Howe (1926), Bonucci (1966) and Thyberg et al. (1971).

Walbach & Howe made specific reference to the fact that the haemopoietic marrow is replaced by a characteristic loose, oedematous-appearing, fibrous or ‘scurvy’ marrow (Frasergerius) containing spindle-shaped stellate fibroblasts. Cells, morphologically osteoblasts, appeared to revert back to fibroblasts so that, in the late stages of acute scurvy, it was difficult to demonstrate them in regions of bone formation. We have not been able to confirm such a ‘reversion’, but our ultrastructural studies have failed to demonstrate the development of cells with the characteristics of osteoblasts. Murray & Kodicek (1949a, b) had shown that no new bone formed within 14 days after fracture in subscorbutic guinea pigs, but the addition of Vitamin C to the diet allowed it to form. This has not been our experience with ectopic bone formation as studied in our particular model. However, the time relationships, and the stimulus to osteogenesis, were different. Perhaps the osteoconductive nature of fracture healing is different to induced osteogenesis.

Our earlier study (1977) reported the formation of new bone after marrow transplantation in less than 14 days, in normal guinea pigs. We indicated the essential role of marrow cells in osteogenesis, and postulated that a response of bone marrow to insult or injury, the formation of bone, is a fundamental biological phenomenon (Cummine & Nade 1976, Nade 1979). Using myelo-osseous grafts, we have looked at the effects of partial Vitamin C deprivation, and made three significant observations.

1. The cellular population at the implant site was unlike that in the normal animal, but similar to that of the ‘scurvy marrow’ of Walbach & Howe, and that gleaned from the reports of Murray & Kodicek (1949a, b, c), Banks (1943) and Holst & Frolich (1907). The ovoid cells described previously (Cummine & Nade 1977) as part of the cell population surrounding implants after 5 days, were rarely seen in any graft in subscorbutic animals. The absence of both ovoid cells and new bone in the subscorbutic guinea pig may well be coincidental; no theory is offered about their relationship. The presence of giant cells in the absence of new bone makes it unlikely that these cells contribute to osteogenesis. It may well be that they are derived from a different precursor cell. The histologic and ultrastructural appearances of cells reflect their metabolic function. In scurvy, general metabolic activity is abnormal, particularly protein synthesis, and cells associated with that function were not often found.

2. There was no formation of bone in the 14-day period studied. Transplanted autologous marrow cells have great ability to produce new bone in heterotopic sites, but can be prevented or delayed from fulfilling this potential. In our myelo-osseous grafts, a deficiency of Vitamin C in the recipient could not have affected the devitalised bone component from another animal. If any ‘osteogenic factor’ resided in such grafts, it would remain unchanged. Only living cells, be they the autografted marrow cells or recipient cells at the implant site, could have their osteogenic potential inhibited. Irrespective of mechanism, the absence of new bone in this study demonstrated the fundamental place of the cell in osteogenesis. The fact that new bone only formed in the presence of bone marrow (Cummine & Nade 1977) strongly indicates that it is cells of the bone marrow which possess the osteogenic potential. The work of Danis (1956) and Friedenstein et al. (1966), supports this conclusion, although this is disputed by Gray & Elves (1979).

3. In an extraskeletal site, using a well-tested model for osteogenesis, Vitamin C deprivation prevented the normal path towards bone formation after bone marrow transplantation. Extracellular synthesis of collagen-like material did occur, but the matrix was abnormal, consisting of a very loosely-woven network of thin fibres. Furthermore, the cell types adjacent to such fibres, and presumably involved in their syn-
esis, did not have ultrastructural characteristics of osteoblasts. Matrix vesicles, usually seen at the junctional area of osteoblasts and osseous matrix, did not appear.

Our three studies combined (Cummine & Nade 1977, Nade 1977, and the present study) indicate the dominant role of marrow cells in osteogenesis and cast further doubt on the primary role of devitalised bone as an inducer of new bone. Indeed, we can find little evidence to support the theory of an 'osteogenin' as a single chemical substance, as initially postulated by LeVander (1941), and pursued by Urist. (See Urist & Dawson 1980) for references to Urist's earlier work.)

We have previously indicated the possibility of 'boneless bone grafting' for clinical use. The osteogenic potential of cells in the bone marrow can be augmented (Sharrard & Collins 1961, Burwell 1966, Boyne 1973, Nade 1979) by providing a suitable environment. This osteogenic potential can also be inhibited as it was in this study. The experiments reported here demonstrated that interference with cell function by deprivation of a single essential molecule, produces great change in the ability of cells to 'synthesise bone' and alters well-described patterns of cell population and morphology. Such a concept is fundamental to our understanding of bone formation, or its aberrations. We suggest that this extraskeletal model for osteogenesis might be a useful tool for studying basic mechanisms of bone formation.

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