THE INFLUENCE OF CORTISONE AND IMPLANTATION SITE ON BONE AND CARTILAGE INDUCTION IN VARIOUS ANIMALS

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When decalcified lyophilised bone matrix (Urist, Silverman, Büring, Dubuc and Rosenberg 1967) is implanted intramuscularly in other animals of the same species, new cartilage, bone and bone marrow appear. The sequence of events leading to this induction and its main features were described in detail by Urist and his co-workers (Urist and Dowell 1968; Urist, Dowell, Hay and Strates 1968; Thompson and Urist 1970). Their view was that after partial resorption of the implanted matrix, endomysial cells dedifferentiated, multiplied and then differentiated into chondroblasts or osteoblasts (Urist 1970).

Recently a further technique for cartilage and bone induction has been described, entailing the use of living cells (Anderson, Merker and Fogh 1964; Wlodarski 1969; Wlodarski, Hinek and Ostrowski 1970). When established tissue culture lines of epithelial cells of both normal and neoplastic origin are grafted intramuscularly, bone and cartilage develop in the adjacent host tissue (Wlodarski 1969, Wlodarski and colleagues 1970). This phenomenon occurs in transplants between animals of different species, though an immunosuppressant must be used to protect the foreign cells (Ostrowski, Wlodarski, Skarzinska and Półtorak 1970; Wlodarski and Hancox 1972), because their survival after grafting is essential for induction. Rather similarly, by using cortisone as an immunosuppressant it was possible for the first time to reveal that the well known osteoinductive properties of transitional epithelium could be effective across the barrier between species (Wlodarski, Półtorak, Zaleski and Ostrowski 1971).

Though the inducing stimuli in these two experimental systems are apparently quite different—that is, a non-viable product obtained from bone matrix on the one hand, and living cells on the other—the end-results after grafting seem very similar if not identical. In the work described below an attempt has been made to find out more about the comparative properties of the two systems, and in particular to try to answer the following questions.
1) Can induction be obtained when Urist’s material is implanted into an animal of a different species? The reduction of the antigenicity of bone which has been shown to follow lyophilisation (Kossowska-Paul 1966) seemed an important factor in this question. 2) Is it necessary to use an immunosuppressant for bone induction to be manifest after such implantation? 3) Does immunosuppressant influence cartilage and bone induction when Urist material is used in animals other than rabbits? 4) Does the actual site of implantation of Urist material influence induction in the same way as do grafts of living epithelial cells?

To answer these questions, the decalcified lyophilised rat bone matrix was implanted into various sites in mice with and without cortisone treatment. It was also implanted into gerbils with and without cortisone treatment and into cortisone-treated rabbits. In order to test the immunosuppressive efficacy of the cortisone dosage in the gerbils and rabbits, living xenogenic grafts of the urinary bladder mucosa of the guinea-pig were used as controls. It seems that this is the first report of the use of gerbils in bone induction experiments: for this reason, and because with them we obtained different results from those with other laboratory animals, it was thought worthwhile to gather more information on their response to osteoinductive stimuli. To that end, human amniotic cells of the tissue culture WISH line and allogenic urinary bladder mucosa were grafted intramuscularly into cortisone-treated gerbils.
Figure 1—Mouse. No cortisone. Forty-six days after intramuscular implantation. Remains of implant (I) at left; induced cartilage (C) in contact with implant and host muscle (M) to right. (x 250.) Figure 2—Mouse. 5 milligrams cortisone. Seventeen days after intramuscular implantation. Remains of implant (I) are below; a mass of induced cartilage and some bone (C, B) has formed on its surface. Host muscle (M) above. (x 155.)

Figure 3—Mouse. 5 milligrams cortisone. Forty-four days after subcutaneous implantation. Implant (I) surface is covered by a small amount of more darkly staining, newly deposited bone (B). This extended over a few serial sections only. (x 250.) Figure 4—Mouse. No cortisone. Eighteen days after intraperitoneal implantation. This graft was found adherent to under-surface of abdominal wall muscle, which can be seen (M) to right. Implant (I) surface is irregular, with indentations, probably formed by post-grafting resorption, now occupied by cartilage (C). (x 150.)

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MATERIALS AND METHODS

Mice (20–26 grammes) of both sexes, of A2G strain or random bred, gerbils (54–67 grammes) of both sexes, and randomly-selected rabbits (960–1,040 grammes) were used in these experiments. Bone matrix material prepared from Sprague-Dawley rats was kindly supplied by Dr Marshall Urist. Samples used were from lot K52171 (defatted for two hours in 1:1 chloroform—methanol, decalcified for sixteen hours in 1 mM DMSO in 1M H₃PO₄ at 2 degrees Celsius, rinsed with distilled water, frozen and lyophilised), and from lot K81970 (decalcified for two days in 0-6 HC1 at 2 degrees Celsius, washed in water, frozen, lyophilised). Under ether anaesthesia, small pieces of the matrix material were implanted intramuscularly into the leg, subcutaneously into the abdominal wall and intraperitoneally, in the mouse; in the rabbit and the gerbil, intramuscular implants only were made. Intramuscular pockets were sutured with catgut; skin wounds were closed with cotton.

Urinary bladders were removed aseptically from guinea-pigs or gerbils, and under ether anaesthesia small pieces of mucosa were grafted to the surface of leg muscle in rabbits and gerbils respectively. These grafts were secured in place with catgut, covered by superficial muscle, and the skin was sutured with cotton.

Cortisone acetate (Cortisyl, Roussel) was given subcutaneously. The dosage schedule is detailed in the tables below which also show the times at which animals were killed. The grafts together with surrounding tissues were then excised, fixed in Bouin’s solution, and decalcified (5 per cent formic acid). Paraffin sections were stained with haematoxylin and eosin and sometimes by the periodic acid-Schiff technique.

RESULTS

MICE

Intramuscular implants—The results with implants of Urist’s material in different sites in mice with or without cortisone treatment are presented in Table I and can be summarised as follows. In a period of two to three weeks, newly-formed hyaline cartilage (Fig. 1) or bone was found around almost every intramuscular implant. These tissues were in direct contact with the implant, very often localised in canals actually within the implant. Bone marrow was often present within the induced tissue.

In mice treated with cortisone (single or repeated doses) the yield of cartilage/bone (Fig. 2) was obviously higher than in control animals without cortisone; in these, the implanted matrix

<table>
<thead>
<tr>
<th>Matrix lot</th>
<th>Cortisone dosage</th>
<th>Length of experiment (days)</th>
<th>Intramuscular Grafts Inductions</th>
<th>Subcutaneous Grafts Inductions</th>
<th>Intraperitoneal Grafts Inductions</th>
</tr>
</thead>
<tbody>
<tr>
<td>K 52171</td>
<td>None</td>
<td>15–24</td>
<td>10</td>
<td>9</td>
<td>1*</td>
</tr>
<tr>
<td>K 5471</td>
<td>None</td>
<td>46</td>
<td>10</td>
<td>9</td>
<td>1*</td>
</tr>
<tr>
<td>K 52171</td>
<td>5 milligrams when implants inserted</td>
<td>17–26</td>
<td>9</td>
<td>8</td>
<td>1*</td>
</tr>
<tr>
<td>K 81970</td>
<td>5 milligrams on day before, and on fourth, tenth and fourteenth day after implanting</td>
<td>17–24</td>
<td>13</td>
<td>13</td>
<td>* Very weak induction (see Fig. 5).</td>
</tr>
<tr>
<td></td>
<td></td>
<td>39</td>
<td>13</td>
<td>13</td>
<td>† Implant adherent to muscle of abdominal wall (see Fig. 4).</td>
</tr>
</tbody>
</table>

TABLE I
RESULTS OF IMPLANTATION IN MICE

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FIG. 5
Mouse. No cortisone. Forty-six days after intraperitoneal implantation. In the centre is a small island of cartilage within a channel in the implant material, which occupies the rest of the field. The cartilage island was extremely tiny and extended over three sections (total, about 25 μm) only. (× 250.)

tended to be infiltrated by mononuclear cells. Six to seven weeks after implantation, the incidence of bone and cartilage was as high, but, in comparison with the findings at two to three weeks, only small amounts of cartilage were present.

Subcutaneous implants—With subcutaneous implants, cartilage and bone were induced, but the incidence was low, especially in control animals. The yield was extremely sparse: induced cartilage or bone was confined sometimes to but a few sections (Fig. 3). No differences were apparent between the control and the cortisone-treated group.

Intraperitoneal implants—The implants were found to have become located in adipose tissue between loops in the gut, except in one case where the graft was adherent to the peritoneal surface of the muscle of the abdominal wall; cartilage had developed within (Fig. 4). In one other case only was cartilage identified in an intraperitoneal implant. The amount was exceedingly small and confined to only three serial sections (Fig. 5). In all cases the matrix implants were invaded by host connective tissue, and the histological picture showed that matrix resorption had taken place. It seemed less extensive in the cortisone-injected animals.

GERBILS

The results with gerbils are set out in Table II. In contrast to intramuscular implants in mice, only slight cartilage inductions were found with gerbils. Histologically there were no

<table>
<thead>
<tr>
<th>Implant</th>
<th>Cortisone dosage (milligrams)</th>
<th>Duration of experiment (days)</th>
<th>Grafts recovered</th>
<th>Number of inductions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Matrix, lot K8 1970</td>
<td>Day before grafting  7-5</td>
<td>18</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Day of grafting  5-0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fourth day after  7-5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Eleventh day after  10-0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Guinea-pig transitional epithelum (xenogenic)</td>
<td>As above</td>
<td>18</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Epithelium did not survive</td>
</tr>
<tr>
<td>Matrix, K8 1970</td>
<td>None</td>
<td>18</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cartilage only</td>
</tr>
<tr>
<td>Allogenic transitional epithelum</td>
<td>Day of grafting  15-0</td>
<td>18</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Fourth day after  10-0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TABLE II
RESULTS OF IMPLANTATION IN GERBILS

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Fig. 6
Gerbil. Cortisone, details in Table II. Eighteen days after intramuscular implantation. A small quantity of cartilage (C) has been induced at one site near the edge of the implant (I). (× 250.)

Fig. 7
Figure 7—Gerbil. Cortisone, details in Table II. Eighteen days after intramuscular grafting of allogenic bladder mucosa. Surviving transitional epithelium (E) covers the right-hand edge. Small islands of bone (B) have been formed in the host connective tissue nearby. (× 250.)

Fig. 8
Figure 8—Gerbil. Same animal as in Figure 7. Note large island of surviving transitional epithelium (E). A small spicule of bone (B) has been induced nearby. (× 150.)
obvious differences between the cortisone-injected (Fig. 6) and the control groups. No induction occurred with grafts of bladder mucosa from the guinea-pig; the transitional epithelium was completely destroyed eighteen days after grafting. However, in three of five urinary bladder allogenic grafts, small amounts of bone were observed. They were confined to a few serial sections only (Fig. 7). There was a very close anatomical relationship of bone to graft. The epithelium survived very well; many islands of epithelial cells and epithelial-lined cysts were present (Fig. 8).

RABBITS

The results of grafting matrix and urinary bladder from the guinea-pig into rabbits injected every day with cortisone (15 milligrams/kilogram of body weight) are presented in Table III.

TABLE III
RESULTS OF IMPLANTATION IN RABBITS

<table>
<thead>
<tr>
<th>Implant</th>
<th>Cortisone dosage</th>
<th>Duration of experiment (days)</th>
<th>Grafts recovered</th>
<th>Number of inductions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Matrix, K8 1970</td>
<td>15 milligrams/kilogram daily</td>
<td>20</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Xenogenic transitional epithelium</td>
<td>As above</td>
<td>20</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>

In two of three rabbits, cartilage induction was found inside the matrix or in very close contact with it. However, the cartilage induction (Figs. 9 and 10) was weak and limited to a few sections only. The cartilage induced in these rabbits had a somewhat atypical histological appearance (Fig. 9). The chondrocytes were more flattened than usual and rather more plentiful than in ordinary hyaline cartilage. The intercellular matrix was more basophil than is usual. The significance of these morphological peculiarities is not clear. Neither bone nor bone marrow-like tissue formation was encountered in the rabbits. The implanted matrix was poorly invaded by host connective tissue; no giant cells were found in the vicinity.

Fifteen days after grafting urinary bladder wall, cysts lined with epithelium were found. In two cases (in one rabbit) small amounts of bone tissue had developed near the epithelium.

DISCUSSION

With Urist's decalcified lyophilised matrix, cartilage and/or bone induction was regularly obtained in transplants between animals of different species, irrespective of the use of cortisone.

The morphological pattern of induction was identical to that obtained by Urist (Urist and colleagues 1967; Urist and Dowell 1968; Urist and colleagues 1968; Urist, Hay, Dubuc and Büring 1969; Urist 1970): mesenchymal cells invaded the matrix canals and were transformed into chondroblasts, osteoblasts or bone marrow cells. Gradually the amount of cartilage decreased and, in later steps of induction, bone tissue was predominant. Very similar events follow the grafting of established epithelial cell lines (Anderson and colleagues 1964, Wlodarski 1969, Wlodarski and colleagues 1970). The main morphological differences seem to be, first, that with living xenogenic cells as inductor, cartilage appears earlier than with matrix. Second, with matrix the induced tissues are located very close to, usually in actual apposition to the implant, either to its outer surface or to surfaces of its canals. They are never separated from it by an envelope of connective tissue as happens with induction by living grafts.
The results indicate a dependence of induction on the site of grafting. Subcutaneous implants of matrix provoked cartilage and bone induction in mice only occasionally and to a much lesser extent than intramuscular. Slightly better results were obtained when cortisone was used. Our results do not confirm reports (Urist and colleagues 1969) that a high incidence of bone induction follows subcutaneous implantation of allogenic matrix. However, they used rats and rabbits as the donors. Again, we (Wlodarski, Półtorak and Koziorowska 1971; Hancox and Wlodarski 1972) have not obtained bone induction when WISH cells were injected subcutaneously into mice or rats, although these cells injected intramuscularly lead to induction.

Such results point again to endomysial cells as a prime target in the inductive system, as has been suggested by Urist and his colleagues (Urist and colleagues 1969, Nogami and Urist 1970). Negative results with matrix implanted intraperitoneally seem to confirm this statement.

Extremely weak cartilage induction was obtained when matrix was implanted intramuscularly into gerbils, irrespective of the use of cortisone. In both control and cortisone-treated groups only very small numbers of chondrocytes and amounts of cartilage matrix were found occasionally inside the old matrix. Also, with the gerbil, bone induction after grafting of allogenic transitional epithelium was weak, although of quite high incidence (three of five animals). Therefore, we suggest that gerbils are much less responsive to the induction stimulus than animals such as mice or rats. The reason for this is quite obscure, but, of course, may be associated in some way with the fact that gerbils are desert animals possessing unusual metabolic properties.
Weak cartilage induction was also obtained when xenogenic matrix was used in rabbits which were treated systemically with cortisone (15 milligrams/kilogram a day). In these same animals, bone induction was observed after grafting of transitional epithelium from the guinea-pig. Bone induction by xenogenic transplants of urinary bladder mucosa in mice, guinea-pigs and hamsters treated with cortisone was described earlier (Wlodarski, Półtorak, Zaleski and Ostrowski 1971) and rabbit can now be added to that list. Ever since the pioneer experiment of Huggins (1931) it has been accepted that, in the rabbit, induction invariably fails to occur around bladder mucosa grafts, even if autogenic. The occurrence of induction by guinea-pig transitional epithelium in our cortisone-treated rabbits suggests that rabbit bladder mucosa lacks a factor present in most other species.

Thompson and Urist (1970) who used identical doses of cortisone and allogenic matrix, observed neither cartilage nor bone induction in rabbits. It is difficult to explain the conflict between our results and those of Thompson and Urist. However, the volume of induced cartilage in our experimental material was exceedingly small, and probably an element of luck was involved in detecting it as tiny though definite areas in a few histological sections. It is possible that it would have been missed with unfavourable planes of section.

Finally, it should be emphasised that, in every experimental system used, cortisone, given even in large, repeated doses, and, in some cases, administered a day before tissue or matrix grafting, has never inhibited cartilage or bone induction in our experiments. This emphasises the usefulness of immunosuppressive agents in work on induction.

### SUMMARY

1. Decalcified lyophilised rat bone matrix prepared by Urist’s method acts as an inductor of cartilage and bone when implanted into animals of other species, namely mice, rabbits and gerbils. Induction in rabbits and gerbils was very much weaker than in the mouse.
2. The site of implantation affected the outcome; intramuscular implants induced cartilage and bone more strongly and regularly than subcutaneous or intraperitoneal implants.
3. Rabbit transitional epithelium, growing in cortisone-treated gerbils, caused bone induction, but in general, results with this species suggest that it responds poorly to bone-inducing stimuli.
4. Cortisone, used as an immunosuppressant, did not inhibit bone and cartilage induction.

### REFERENCES


THE INFLUENCE OF CORTISONE AND IMPLANTATION SITE ON BONE AND CARTILAGE INDUCTION


