Alternatives to autogenous bone graft for spinal fusion have been investigated for many years. It has been shown that osteoconductive materials alone do not give a rate of fusion which is comparable to that of autogenous bone graft. We analysed the effectiveness of porous ceramic loaded with cultured mesenchymal stem cells as a new graft material for spinal fusion in an animal model.

Posterolateral fusion was carried out at the L4/L5 level in 40 White New Zealand rabbits using one of the following graft materials: porous ceramic granules plus cultured mesenchymal stem cells (group I); ceramic granules plus fresh autogenous bone marrow (group II); ceramic granules alone (group III); and autogenous bone graft (group IV). The animals were killed eight weeks after surgery and the spines were evaluated radiographically, by a manual palpation test and by histological analysis.

The rate of fusion was significantly higher in group I compared with group III and higher, but not significantly, in group I compared with groups II and IV. In group I histological analysis showed newly formed bone in contact with the implanted granules and highly cellular bone marrow between the newly formed trabecular bone. In group II, thin trabeculae of newly formed bone were present in the peripheral portion of the fusion mass. In group III, there was a reduced amount of newly formed bone and abundant fibrous tissue. In group IV, there were thin trabeculae of newly formed bone close to the decorticated transverse processes and dead trabecular bone in the central portion of the fusion mass.

In vitro cultured mesenchymal stem cells may be loaded into porous ceramic to make a graft material for spinal fusion which appears to be more effective than porous ceramic alone. Further studies are needed to investigate the medium- to long-term results of this procedure, its feasibility in the clinical setting and the most appropriate carrier for mesenchymal stem cells.

Posterolateral fusion with autogenous bone graft is commonly used in patients with developmental or degenerative spinal conditions which are unresponsive to conservative therapy. It has been shown that the procedure is safe and that, in properly selected patients, high rates of bony fusion and successful clinical outcomes can be obtained.1-3 However, several limitations, including pseudarthrosis, pain at the donor site, the need for blood transfusion and prolonged operating times are known to be associated with the procedure.4-7

Alternatives to autogenous bone graft to achieve spinal fusion have been investigated over many years.8-14 It has been shown that bone morphogenetic proteins (BMPs) with adequate carriers may achieve similar, or even higher, rates of fusion compared with posterolateral fusion with autogenous bone graft.8,10,12,15 However, the cost of the dose of BMPs which is required is high and there are possible adverse effects of these growth factors.

Porous ceramic cylinders loaded with cultured mesenchymal stem cells have been tested as an alternative to autogenous bone graft in critical-sized defects of long bones.16 The results have suggested that healing of segmental bone defects was more likely to occur with porous ceramic loaded with cultured mesenchymal stem cells than with porous ceramics alone.16 Since the healing of a critical-sized defect of long bones is considered to be more challenging than that of other conditions, such as bone cavities, we hypothesised that porous ceramic loaded with cultured...
mesenchymal stem cells could be used successfully even in an unfavourable environment such as the graft bed in posterolateral spinal fusion.

Materials and Methods

Posterolateral fusion was performed in 40 adult White New Zealand rabbits weighing 4.5 kg. The animals were randomly allocated to receive one of the following graft materials: a) porous ceramic granules plus cultured mesenchymal stem cells (group I); b) ceramic granules plus fresh autogenous bone marrow (group II); c) porous ceramic granules alone (group III) and d) autogenous bone graft (group IV).

The surgical procedure was the same in all animals. Briefly, under general anaesthesia, through a dorsal midline skin incision two paramedian fascial incisions were made and through the intermuscular plane between multifidus and longissimus the transverse processes of the fourth and fifth lumbar vertebrae were exposed on both sides. With a small rongeur the transverse processes were decorticated and 2 g of graft material were positioned on the graft bed on each side.

Graft materials

Group I. Porous ceramic granules and cultured mesenchymal stem cells were used. The procedure included the following phases.

Collection of bone marrow. After preparation of the skin and under general anaesthesia (10 mg/kg of intramuscular ketamine and 20 mg/kg of intravenous sodium pentobarbiturate), 8 to 10 ml of bone marrow were aspirated from one or both iliac crests with an 18-gauge needle. The material was placed in heparinised tubes and sent for culture in vitro.

Culture and differentiation of mesenchymal stem cells. The bone marrow was mixed with one volume of phosphate-buffered saline and the nucleated cell fraction was enriched for mesenchymal stem cells by density-gradient centrifugation over a Lympholyte-H (Cedarlane, Hornby, Ontario, Canada) cushion. The cells at the medium-Lympholyte-H interface were collected, washed three times with culture medium (DMEM/HAMS F-12 (Dulbecco’s MEM/Nutrient Mix F12), and seeded into culture flasks with a standard culture medium which consisted of DMEM/HAMS F-12 supplemented with 15% fetal calf serum, 25 mM HEPES, 50 µg/ml of gentamicin, 2 µg/ml of amphotericin B and 50 µg/ml of L-ascorbic acid. The cells were harvested at 37°C. On the sixth day of culture, the non-adherent cells were removed along with the culture medium. The medium was changed twice weekly. Confluent monolayers were obtained about one week after the start of cell division. Mesenchymal stem cells were subcultured at 10 to 14 days. To obtain subpassages, cell monolayers were recovered by digestion with 0.25% trypsin-verseine solution. Before treatment with trypsin, careful washing of monolayers with phosphate-buffered saline was performed to eliminate trypsin inhibitors. Subcultivation was performed by replating at 6 x 10⁵ cells per cm² flasks. For each passage, one control culture was performed.

To promote osteogenic differentiation, cells were stimulated during subpassages by cultivation in standard medium supplemented with 100 nM dexamethasone. At the completion of cell culture, the medium was removed, the cell layer washed in phosphate-buffered saline and digested as previously described. Samples of cell suspension were diluted with 0.5% Trypan Blue and the number of viable cells determined. An evaluation of the activity of alkaline phosphatase by an enzymic immunoassay technique was carried out to assess the osteogenic differentiation of cell cultures. Morphological examination on the stained monolayered cells and histochemical evaluation using the Von Kossa method were also performed.
Loading of ceramic granules with mesenchymal stem cells. Reabsorbable granules of calcium phosphate (80%) and hydroxyapatite (20%), with a dimension of 1 to 4 mm and a pore size ranging between 200 and 400 µm (Pro-Osteon 500R, Irvine, California) were used. Briefly, once approximately 30 million cells had been obtained from the in vitro culture, the porous ceramic granules were soaked in a solution containing 100 mg/ml of fibronectin (Fibronectin cellular from human foreskin fibroblast; Sigma-Aldrich, Milan, Italy) for 16 hours at 4°C. This procedure was found to be necessary since, without it, little cell adhesion occurred on the surface and within the pores of the granules (Fig. 1). The solution was then discharged and the granules were placed in 5 ml of cell suspension in culture medium at 37°C and gently agitated for three hours to facilitate the flow of cell suspension into the pores. A number of residual cells, approximately 10% of the initial cell population, were found in the suspension medium after the removal of ceramic granules.

Implantation of the graft material at surgery. Under general anaesthesia, animals from which bone marrow had been collected underwent spinal fusion as previously described. Once the graft bed had been prepared, 2 g of porous ceramic granules loaded with mesenchymal stem cells were implanted as graft material on each side.

Group II. Porous ceramic granules and fresh bone marrow were used. Before implantation, the ceramic granules were soaked in a solution of fibronectin as in group I. Under general anaesthesia the graft bed was prepared and 2 g of ceramic granules were placed on the decorticated transverse processes. Then 10 ml of bone marrow were aspirated from one or both iliac crests with an 18-gauge needle and immediately placed on the graft material (5 ml on each side).

Group III. Porous ceramic granules alone were used. Before implantation, they were soaked in a fibronectin solution as in groups I and II.

Group IV. Autogenous bone graft was harvested from both iliac crests (2 g of corticocancellous bone graft on each side) and used as graft material.

Post-operative treatment. All the animals were housed and treated according to university-approved guidelines. Antibiotics and analgesic medications were given from the first to the third post-operative day. The animals were able to move on the first post-operative day and gained weight between the first and second week after surgery.

Assessment of fusion. The animals were killed eight weeks after surgery by an intraperitoneal injection of phenobarbital. The lumbar spine was harvested en-bloc and the fusion mass was evaluated radiographically, by a manual palpation test and by histological analysis.

Radiographic evaluation. Anteroposterior plain films of the lumbar spine were evaluated blindly by two of the authors (GC, GG) according to the subjective grading scale used by Curylo et al. In summary, a radiographic score of four points was given when a continuous bony mass was present on both sides without lucency, three points when a continuous bony mass was present bilaterally with lucency on one side, two points when a bony mass was present on both sides with lucency bilaterally, one point when a bony mass was present on one side only and zero points when a bony mass was not seen on either side. A solid fusion was diagnosed when the radiographic score was three or four and pseudarthrosis when the score was two or less. On axial CT (2 mm slice thickness) the presence of newly formed bone between ceramic granules and, in the control group, between corticocancellous bone chips, was evaluated.

Manual palpation test. After the division of the supraspinous and interspinous ligaments, the posterior joints and the intervertebral disc were excised, thus leaving the graft material as the only tissue connecting the adjacent vertebrae. This was performed at the operated level and, for comparison, at the adjacent non-operated levels above and below. The result of the manual palpation test was classified as solid fusion, when no vertebral movement was detected at the operated level and, as uncertain, when vertebral movement was present at the operated level but markedly reduced compared with the adjacent levels. When vertebral movement was equal to, or slightly reduced, compared with the adjacent levels, the result was classified as a pseudarthrosis.

Histological analysis. Specimens including the newly formed bony mass and the transverse processes were fixed, at least overnight, in 4% formaldehyde freshly prepared from paraformaldehyde in phosphate buffer at pH 7.2 and then cut longitudinally into macroscopic consecutive sections 5 mm thick. One of the most representative sections was decalcified and routinely processed for paraffin embedding. Another was embedded in glycolmethachrylate without decalcification. Serial sections 5 µm and 2 µm thick were cut from paraffin blocks and from plastic blocks, respectively. The sections were stained with haematoxylin and eosin (HE), and azure II-Methylene Blue and examined under light microscopy.

Statistical analysis. Non-parametric tests were used for statistical analysis. In particular, the Kruskal-Wallis test was used to evaluate the overall differences between the four groups and the Mann-Whitney test to analyse differences between each group of treatment. A p value of less than 0.05 was considered to be statistically significant.

Results

The surgical procedure was well tolerated in all groups. In group I, two rabbits died as a result of anaesthetic complications and one developed a deep infection. In group II, one animal died from anaesthetic complications and one developed a subcutaneous infection which did not involve the graft material. Two animals died in group IV, one from anaesthetic complications and one from a gastrointestinal infection four weeks after surgery.

Gross inspection. In groups I, II and III, the implanted granules were visible in the external aspect of the fusion mass and...
variously incorporated within it. In specimens of group I and II ceramic granules were tenaciously adherent to the surrounding tissue, while in group III they were less adherent to, and more easily detachable from, the adjacent tissue compared with groups I and II. In all specimens of groups I, II and III, ceramic granules showed evidence of partial resorption, i.e., they were usually smaller and had round edges compared with their initial size and shape, and pores were less evident than initially. No specimen showed signs of inflammation around the implanted material on gross inspection.

Radiological findings. These are given in Table I and Figure 2. There was a significant difference between the percentage of solid fusion observed in the four groups (p = 0.039). In group I the rate of fusion was significantly higher than that in group III (p = 0.015) and higher, but not significantly, than that in group IV (p < 0.06). No significant difference was found between groups I and II (p = 0.06) and between group II and groups III and IV.

Histological analysis

Group I. Newly formed bone was observed in contact with the implanted granules (Fig. 3a). In many specimens the entire surface of the granules and pores was in contact with the newly formed bone, while in a few the surface of the granules was in contact with tight fibrous tissue. Most of the new bone appeared to be without cartilaginous formation, although islands of cartilaginous tissue and endochondral ossification were also present (Fig. 3b). Highly cellular bone marrow was found between newly formed trabecular bone. The remodelling processes included appositional bone formation with active osteoblasts aligned on the surface of bone trabeculae.

Group II. New trabecular bone was observed adjacent to the implanted granules. There were several differences from group I. First, the apposition of new bone was more abundant in the peripheral portion of the fusion mass than in the

**Table I.** The radiological findings. A score of 3 + 4 corresponds to solid fusion. The total number of specimens is given in parentheses

<table>
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<th>Group</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>3 + 4</th>
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<tbody>
<tr>
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</table>

**Table II.** The results of the manual palpation test. The total number of specimens is given in parentheses

<table>
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<tr>
<th>Group</th>
<th>Solid fusion</th>
<th>Uncertain fusion</th>
<th>Pseudarthrosis</th>
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<tr>
<td>I</td>
<td>4</td>
<td>3</td>
<td>0 (7)</td>
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<tr>
<td>II</td>
<td>3</td>
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<td>III</td>
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</tr>
<tr>
<td>IV</td>
<td>2</td>
<td>2</td>
<td>4 (8)</td>
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</table>

Manual palpation test. The results of the manual palpation test are given in Table II. In group I, the rate of pseudarthrosis was significantly lower than that in group III (p = 0.015) and lower, but not significantly, than that in group IV (p ≤ 0.06). No significant differences were found between the other groups.
central portion. The new trabecular bone appeared to be thinner compared with that of specimens of group I, and bone marrow between trabecular bone showed a reduced number of nucleated cells.

**Group III.** A reduced amount of new bone was present compared with the other groups. The newly formed bone was mainly located close to the transverse processes. Fibrous tissue was often present adjacent to the ceramic (Fig. 3c). Scant haematopoietic marrow tissue was present.

**Group IV.** Thin trabeculae of newly formed bone were present close to the decorticated transverse processes and, more rarely, in the central portion of the graft bed. Dead trabecular bone showing no evidence of revascularisation was frequently seen at the central portion of the fusion mass (Fig. 3d).

The quantitative differences in the amount of bone formation between group I and the other groups were particularly evident in the central areas of the graft. In such areas a prevalence of fibrous tissue was seen in the experimental groups to which mesenchymal cells had not been added, while newly formed bone was present in group I. In the peripheral portion of the graft, the quantitative difference in bone formation was evident between groups I and II compared with groups III and IV. In the latter, formation of new bone was only present close to the transverse process. Differences between group I and group II were less pronounced in the peripheral portion of the graft. They were mainly due to the thinner conformation of the bony trabeculae in group II.

**Discussion**

Autogenous bone graft is known to supply the necessary materials to promote the formation of new bone including bone-forming cells, growth factors and a scaffold to support bone cells and intercellular matrix. As a result, autogenous
bone graft has been successfully used even in challenging procedures, such as arthrodesis of the spine, in which a brace or plaster cast was the only device used to stabilise the graft material. However, in specific surgical procedures, such as spinal fusion at multiple levels or revision surgery in which bone graft has already been harvested at the time of the initial operation, the amount of autogenous bone graft may be insufficient to promote bony fusion and a pseudarthrosis may occur. Further limitations of autogenous bone graft are the prolonged operating time and the increased perioperative and post-operative morbidity associated with the surgical procedure.

Substitutes of autogenous bone graft for spinal fusion have been investigated for many years. However, some of these studies provided little information since different animal models were used. More recently, an animal model for posterolateral fusion yielding rates of fusion comparable with the clinical setting has been developed. Using this model, materials with osteoconductive properties have been tested, including carriers with bony or porous structures (allograft, porous ceramics, sintered bovine bone) or without bony structures (collagen sheet or polylactic acid polymer). The results of these studies have shown that osteoconductive materials do not give a high rate of fusion when used alone in the posterolateral fusion model. The addition of osteoinductive substances, particularly BMPs, led to a significantly higher rate of fusion than was seen with osteoconductive materials alone. However, the high manufacturing cost and possible adverse effects of BMPs, including the release of factors which may stimulate the production of osteoclasts, may limit the clinical use of these substances. In addition, when BMPs were used in clinical trials, the results were not better than those obtained with autogenous bone graft or demineralised bone matrix was used.

In our present study we investigated the effectiveness of a new graft material for spinal fusion consisting of an osteoconductive carrier loaded with cultured mesenchymal stem cells. The latter are capable of giving rise to bone, cartilage, muscle and fat once exposed to different growth conditions. Since osteogenic differentiation of mesenchymal stem cells has been found to occur in vitro in response to various bioactive factors, we hypothesised that cultured mesenchymal stem cells could provide adequate osteoinductive material for the carrier, either by inducing formation of new bone or by releasing osteogenic growth factors which could stimulate the recruitment and differentiation of further mesenchymal stem cells.

The cultured cells were loaded into ceramic granules, with a pore size ranging from 200 to 400 µm. This material appeared to be a suitable carrier for mesenchymal stem cells since approximately 90% of the initial cell population was found to adhere to the ceramic. However, it was interesting to note that cell adhesion to both the external surface and inner pores of the ceramic was observed only when the granules were presoaked in a solution with fibronectin. This extracellular matrix glycoprotein may play a relevant role in the process of the formation of new bone since it has been shown to regulate the adhesion and migration of mesenchymal stem cells and affect the differentiation and survival of osteoblast-like cells.

A rate of fusion ranging between 50% and 70% and between 0% and 100% has been reported, respectively, when autogenous bone and alternative graft materials were used in experimental posterolateral fusion. In our study, autogenous bone graft gave a rate of fusion comparable with that of previous investigations. Cultured mesenchymal stem cells loaded into porous ceramic achieved a significantly higher rate of solid fusion compared with porous ceramic alone, and a trend towards a higher rate than with autogenous bone. Although the radiographic results may be affected by the radiopacity of non-reabsorbed ceramic, which may render it more difficult to diagnose the presence of pseudarthrosis, both plain radiography and CT showed that even when a continuous bony mass was present, it appeared to be more homogenous and ceramic granules were more adherent to the transverse process in rabbits treated with ceramic and mesenchymal stem cells than in those which had ceramic alone. This result was also confirmed on gross inspection, which showed that in the animals treated with ceramic alone, porous granules were more easily detachable from the adjacent tissue of the fusion mass than in those treated with ceramic plus mesenchymal stem cells or plus fresh bone marrow.

The osteoinductive properties of fresh bone marrow have been documented in studies using a model of a long bone defect. However, when fresh bone marrow was used as osteoinductive material in experimental posterolateral fusion the results were controversial. A comparison of the osteogenic potential of fresh bone marrow and mesenchymal stem cells has shown that the latter induced faster and more extensive formation of new bone than fresh bone marrow. In our study, rabbits treated with ceramic plus mesenchymal stem cells showed a higher rate of fusion than those treated with ceramic and fresh bone marrow but the difference was not significant, probably because of the small number of animals included in each treatment group. However, evident differences between the two groups were found on histological analysis. In particular, the newly formed bone showed thicker trabeculae and more abundant bone-marrow cells in animals treated with ceramic and mesenchymal stem cells than in those treated with ceramic and fresh bone marrow. Moreover, in the latter group, the new bone was essentially present in the peripheral portion of the fusion mass, while in the animals treated with ceramic and mesenchymal stem cells it was seen in both the peripheral and inner portion of the fusion mass. It can be speculated that osteogenic growth factors, which are essential to promote differentiation of bone-marrow cells into osteogenic cells, are likely to be present in the peripheral portion of the graft bed while their presence may be inadequate in the inner portion, probably
because of insufficient blood supply in this area. Conversely, mesenchymal stem cells, which have been stimulated to differentiate in vitro into osteoprogenitor cells, could be less dependent on local growth factors in inducing the formation of new bone.

The results of our study seem to encourage further investigations on the use of mesenchymal stem cells loaded on porous ceramic as graft material for spinal fusion. However, several factors which may affect the success of the procedure should be considered. First, in the clinical setting, mesenchymal stem cells may show different capabilities of growth and differentiation depending on constitutional factors and donor age, and therefore the procedure may be unsuitable for some patients. Secondly, the degree of differentiation of mesenchymal stem cells which is more likely to promote the formation of new bone in the host tissue, is not well known. In particular, it has been hypothesised that less differentiated mesenchymal stem cells, which have greater potential for division and differentiation than more highly differentiated mesenchymal cells, could be more effective in promoting the formation of new bone. Thirdly, the survival of the implanted mesenchymal stem cells may be affected by several factors, including the type of carriers, local conditions of blood supply or other factors which at present are not known.

Finally, according to the manufacturer’s indications, the ceramic carrier used in our study should undergo reabsorption after six months. However, since in this study, the animals were killed eight weeks after the implantation of the ceramic, our results do not provide information regarding the long-term behaviour of the graft material, in particular, whether its complete reabsorption occurs and, if this is the case, whether the reabsorbed ceramic are replaced by bone or fibrous tissue.

In conclusion, our study has shown that mesenchymal stem cells, which have been stimulated to differentiate into preosteogenic cells in vitro, may be loaded into porous ceramic to give rise to a graft material which may promote the formation of new bone in experimental spinal fusion. This graft material appeared to be more effective than porous ceramic alone and at least as effective as autogenous bone graft. Further studies are needed to investigate the medium- to long-term results of this procedure, its feasibility in the clinical setting and the most appropriate carrier for mesenchymal stem cells.

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

References


