Posterolateral lumbar fusions in athymic rats: characterization of a model

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Abstract

BACKGROUND CONTEXT: The athymic rat has been used to study the role of osteoinductive products in spinal fusions. This small animal model has been advocated to minimize potential inflammatory responses to allogeneic or xenogenic proteins. Despite past experience, this model has not yet been well characterized.

PURPOSE: To further define and validate a posterolateral lumbar fusion model in the athymic rat.

STUDY DESIGN/SETTING: Comparison of fusions after animal survival surgery.

PATIENT SAMPLE: Forty athymic and 20 normothymic rats.

OUTCOME MEASURES: Manual palpation, radiography and histology at 3 and 6 weeks.

METHODS: Single-level intertransverse fusions were performed at the L4-L5 level of 40 athymic rats. Twenty rats were implanted with autograft (athymic/autograft), and 20 had no graft placed (athymic/no graft). An additional 20 autograft fusions were performed on normothymic rats (normothymic/autograft). Half were sacrificed at 3 weeks; half were sacrificed at 6 weeks.

RESULTS: At 3 weeks, 0% of the athymic/no graft rats fused, 20% of the athymic/autograft rats fused and 20% of the normothymic/autograft rats fused by manual palpation. At 6 weeks, 0% of the athymic/no graft rats fused, 30% of the athymic/autograft rats fused and 40% of the normothymic/autograft rats fused by manual palpation. Radiographs were of limited utility in determining fusion, and histology results were roughly concordant with those of manual palpation.

CONCLUSIONS: This work further characterizes the athymic rat posterolateral lumbar fusion model. The absence of a thymus does not appear to affect autograft fusion rates, and no spontaneous fusions were seen when no graft was placed. © 2004 Elsevier Inc. All rights reserved.

Keywords: Rats; Nude rats; Animal models; Spinal fusion; Bone transplantation; Lumbar vertebrae

Introduction

Spinal fusion is a common surgical procedure, and autograft is the gold standard grafting material. However, despite advances in surgical techniques, pseudarthrosis remains an issue [1]. Further, autograft may be limited in supply, and its harvest is associated with significant morbidity and increased operative time [2]. Attention has thus been directed toward refining alternative means of inducing bone formation.

Bone graft substitutes that are currently being studied include demineralized bone matrices (DBMs), individual recombinant bone morphogenetic proteins (BMPs) and autogenous blood product isolates. In addition to the active agent in these new products, the mode of delivery and type of carrier are important factors in determining efficacy.

With the many variables being studied, it has been necessary to use animal models. Noninstrumented posterolateral lumbar fusions are commonly studied, because they pose a challenging and clinically relevant model. Studies have been done in a number of species, including rats [3,4], rabbits [5,6] and dogs [7,8]. Products are often tested in lower
species and moved along to higher species only when promising results are found.

Some osteoinductive products, however, cannot be tested in certain models. For example, Aspenburg et al. [9] found xenogenic DBM to induce little or no bone formation when placed intramuscularly in normal rats. It was hypothesized that this was because of an immunologic response of the host to the nonconserved or differentially expressed donor proteins. Substantiating this hypothesis, increased bone induction was seen when athymic recipient rats were studied under otherwise identical circumstances. Although xenogenic DBMs are not used clinically, there is interest in studying them in animals as products are being developed. Other studies have shown human DBM to have a dose-dependent osteoinductive effect when placed intramuscularly or subcutaneously in athymic rats [10].

Additional work is ongoing to study the effectiveness of BMP containing adenoviral vectors (Ad) in inducing bone formation. When Ad-BMP-2 was injected intramuscularly into normothymic rats, significant immune response (without concomitant bone formation) was elicited by the first-generation adenoviral constructs [11,12]. Conversely, bone formation without such immune response was seen when the same construct was studied in athymic rats.

Subsequently, the athymic rat has been used to study the potential role of osteoinductive products in spinal fusions by several authors. Athymic rat fusions have been studied with open implantation of xenogenic DBMs [4,13], injection of adenoviral constructs [14,15] and application of mammalian expression vectors [3].

Much evidence suggests that nonsteroidal anti-inflammatory agents can adversely affect fusion rates [16,17]. Should a similar effect be expected if fusions are studied in the athymic rat where the immune system is compromised? Contrary to what might be expected, bone formation and metabolism does not appear to be affected by the absence of the thymus gland in rats [18]. Another study showed no significant differences in the healing of osteotomized tibias of athymic and normothymic rats [19].

Some small animal models have high fusion rates solely because of surgical exposure. This is not expected in the athymic rat model. Entire study groups have been described by Wang et al. [4] with no fusion (namely with Dynagraft [Citagenix, Inc., Lavai, QC, Canada], which was thought to be of insufficient osteoinductivity). By inference, fusion does not appear to be induced by surgical approach alone. This, however, has not yet been demonstrated as an independent variable.

Although used in previously reported studies, the model for open posterolateral lumbar fusion in the athymic rat has not, to our knowledge, been well characterized as performed for other fusion models [5]. The purpose of the current study is to clearly characterize a posterolateral lumbar fusion model in the athymic rat. Radiographic, manual palpation and histologic examinations are to be used to define the rates of autograft fusion, effect of an absent thymus and rates of spontaneous fusion in this model.

Materials and methods

Overview

Sixty L4-L5 posterolateral lumbar fusions were performed (of note, rats have six lumbar vertebrae). Forty were mature female athymic nude rats (nu/nu), 8 to 9 weeks of age. Twenty received autograft (experimental study group, athymic/autograft). Twenty were opened and closed with no graft placed (negative control group, athymic/no graft). An additional 20 fusions were performed on 9- to 10-week-old immunologically intact female Sprague Dawley rats. These animals were similar to the athymic rats being tested in terms of age and size and were grafted with autogenous bone (positive control group, normothymic/autograft).

For each of the three study groups, sacrifices were performed at two time points: 3 and 6 weeks. This was based on work from Wang et al. [4] in which the maximum number of fusions was observed by 6 weeks when 2-, 4-, 6- and 8-week time points were studied with athymic rat posterolateral lumbar fusions. After sacrifice, fusions were analyzed with radiographs, manual palpation and histology.

Surgical procedure

Our university’s animal care and use committee approved the following protocol. All rats were obtained from Harlan Sprague Dawley, IN, and were acclimated at our facility for a minimum of 1 week before surgery. Surgical procedures were performed for each study group in a mixed, but not truly randomized, fashion.

Rats were induced with ketamine and xylazine (90 mg/kg and 4 mg/kg intraperitoneal, respectively), and anesthesia was maintained with isoflurane (0.5% to 2%) and oxygen by means of a co axial nose cone. Perioperative antibiotics were given (enrofloxacin 10 mg/kg subcutaneously). The rats were positioned and prepared in standard surgical fashion.

L4-L5 posterolateral fusions were then performed with the assistance of loupe magnification. The spine was approached through a single midline skin incision and two paramedian fascial incisions (Witsel approach). The level was identified during surgery by referencing from the iliac crests. Once exposed, the transverse processes were decorticated with a sharp elevator until a blush of cancellous bone was observed.

Autograft study groups

For the athymic/autograft and normothymic/autograft study groups, bone graft was then harvested from both iliac crests. Through the fascial incisions noted above, the iliac crests were exposed. A rongeur was then used to harvest approximately 0.1 to 0.2 cc of corticocancellous bone from
each iliac wing. This bone was morcelized in the process of harvesting, as done with previously described animal fusion models [6]. The wounds were irrigated, and the harvested graft was placed into the fusion beds.

No graft study group

For the athymic/no graft group, the surgical procedure was identical to that described above, except no graft harvest was performed.

Once the graft material was placed as indicated, the fascia was closed with 3.0 vicryl sutures, and the skin was closed with staples. Wounds were dressed, and the animals recovered. For postoperative pain, one dose of Buprenex (Reckitt Benckiser Pharmaceuticals Inc., Richmond, VA) was given (0.03 mg/kg) once the rats were sternally recumbent. Enrofloxacin (Bayer Corporation, Shawnee Mission, KS) (50 mg/ml) and Tylenol (McNeil-PPC Inc., Fort Washington, PA) (1 mg/ml) were administered in the drinking water for the first 2 postoperative days. Dressings were removed after postoperative day 1. As noted in the overview section above, half of the rats were sacrificed at 3 weeks and half at 6 weeks.

Fusion analysis

Posterior-anterior radiographs were taken once the rats were sacrificed (lateral radiographs were not done because the intertransverse regions were overshadowed by the vertebral bodies). Two reviewers evaluated the radiographs for fusion in a blinded fashion. Fusion was determined if bridging bone was noted in either intertransverse region. If there was disagreement in the reading of fusion by the two reviewers, a third reviewer evaluated the films to make the final determination of fusion.

The spines were then explanted, and manual palpation testing of the L4-L5 segment was performed. This has been found to correlate well with more rigorous biomechanical testing in prior rabbit studies [6]. Furthermore, because of the small nature of the rat spines, such biomechanical testing was not possible in this model.

Finally, the L4-L5 spinal segments were embedded in methylmethacrylate (MMA) for histologic evaluation. These specimens were fixed in 70% ethanol, dehydrated in graded ethanol and cleared in toluene under vacuum and pressure on a tissue processor (VIP 2000; Tissue Tek; Miles, Elkhart, IN). The undecalciﬁed specimens were then inﬁltrated with increasing concentrations of MMA and embedded in MMA, according to the method described by Baron et al. [20]. Four-micron coronal sections were deplastriﬁed and then stained with toluidine blue (pH 3.7). Histologic fusion was deﬁned as bony trabeculae bridging from one transverse process to the next.

Statistics

Fusion success rates of the different experimental groups, as determined by manual palpation and histology, were compared using the Fisher exact test. Interobserver reliability kappa values were calculated where appropriate. Results were considered signiﬁcant at p<.05.

Results

The athymic rats used in this experiment weighed between 170 and 200 g. The normothymic rats weighed between 190 and 200 g. The surgical procedures took approximately 20 to 30 minutes to perform.

Three rats were excluded from the study because of complications: two were lost perioperatively because of anesthesia, and one was lost approximately 1 week postoperatively with no clear cause. Of the three rats lost, all were normothymic. These three rats were replaced and are not included in the animal numbers quoted in the methods section. The remainder of the rats in the study were without complications.

Manual palpation of the explanted spines revealed fusion rates as noted in Table 1. At 3 weeks, the normothymic/autograft group had a fusion rate of 20% and the athymic/autograft group had a fusion rate of 20% (difference insignificant with p=1.0). At 6 weeks, the normothymic/autograft group had a fusion rate of 40% and the athymic/autograft group had a fusion rate of 30% (difference insignificant with p=1.0). The athymic/no graft group had no fusions at either time point.

Radiographs revealed no conclusive fusions in the normothymic/autograft or athymic/no graft groups (Fig. 1). One animal was interpreted as fused in the 6-week athymic/autograft group. Of the 60 films reviewed, there was disagreement in reading by the first two reviewers for 5 of the films. Four of these 5 were deemed to be not fused and 1 to be fused by the third reviewer. This yielded a kappa value of 0.24 with an asymptotic (two-tailed) p value of .058 and an exact p value of .19. Overall, with manual palpation as the gold standard for fusion, radiographs were 13% sensitive and 100% specific for fusion in this study. As with other animal and clinical studies, the value of this observation may be of limited value because of the small number of fusions noted and difficulty in detecting fusion radiographically.

Histological analysis at both 3 weeks and 6 weeks revealed no fusions for the athymic/no graft group but a 10% fusion rate for the athymic/autograft group and a 20% fusion rate for the normothymic/autograft group at both 3 weeks and 6 weeks (difference insignificant with p=1.0 for each time interval). The autograft specimens that were interpreted

| Table 1 |
| Rates of fusion as determined by manual palpation |
|-----------------|-----------------|-----------------|
|                 | Normothymic rat | Athymic rat |
|                 | autograft       | Autograft      | No Graft      |
| 3 weeks         | 2/10 rats (20%) | 2/10 rats (20%)| 0/10 rats (0%)|
| 6 weeks         | 4/10 rats (40%) | 3/10 rats (30%)| 0/10 rats (0%)|
as not fused revealed evidence of corticocancellous bone islands (corresponding to the original grafting material) without bridging between the transverse processes. The fused specimens demonstrated clear bridging trabeculae from one transverse process to the next, incorporating the bone graft chips within the fusion mass. There was no interposed fibrous tissue in these specimens. In general, more abundant bone formation was seen adjacent to the transverse processes than in the intertransverse process interval (Fig. 2).

The reliability between manual palpation testing and histologic analysis was studied statistically. For the athymic autograft group, the kappa value was 0.50 with an asymptotic p value less than .01 (two tailed) and an exact p value of .053 (both one and two tailed). For the regular autograft group, the kappa value was 0.47 with an asymptotic p value of .03 (two tailed) and an exact p value of .06 (both one and two tailed). For the athymic no graft group, there was 100% agreement between manual examination and histology, so a kappa value was unnecessary.

Discussion

The athymic rat model has previously been used to study spinal fusion, but additional characterization was required before continuing study with this useful model. In what frame of reference should posterolateral fusion studies performed in these animals be interpreted?

The rat is a small and easily handled animal in which posterolateral fusion is easy to perform. Although much smaller than the human, the morphology of the rat lumbar vertebrae are roughly similar to that of the human (Fig. 3).
Athymic animals offer the potential advantage of minimizing immunologic responses to certain implanted products. Furthermore, complications were found to be minimal with this model (5% as opposed to 20% to 23% with the New Zealand White rabbit model) [5,6].

There were differences in fusion rates between the methods of analysis studied. Manual palpation has proven to be predictive of precise multidirectional biomechanical testing in prior studies [6]. Comparison of manual palpation and histology results demonstrated a high degree of agreement, as shown by the kappa value calculations. Histology provides a precise means of studying bone formation and quality, but it is prone to miss bridging bone if it crosses out of the sectioned plane being studied. For these reasons, we used manual palpation as the standard by which fusion was determined in this study. Radiographs are notoriously poor at determining the presence or absence of fusion in animal [5,6] and clinical studies [1]. Because there is difficulty in detecting fusion with radiographs, no weight was placed on this mode of analysis.

Based on manual palpation results, there was an increase in fusion rate for the autograft study groups between the 3- and 6-week time points studied. This confirms that spinal fusions in the rat should be observed to at least the 6-week time point. As cited earlier, Wang et al. [4] found the maximum number of fusions by this time point.

Human posterolateral lumbar fusions have pseudarthrosis rates reported up to 26% [1]. In the rabbit, posterolateral fusions have pseudarthrosis rates of 33% to 37% [5,6]. This study defines fusion rates for iliac crest autografted posterolateral fusions in the athymic rat for the first time. This rate was found to be 30% at 6 weeks, yielding a pseudarthrosis rate of 70%. Furthermore, no spontaneous fusions were noted in this model, suggesting that any fusions are the result of the osteogenic, osteoconductive and/or the osteoinductive nature of bone graft materials.

Of significant note, the athymic and normothyMIC study groups had statistically equivalent fusion rates. Although this is consistent with reported bone healing responses in these animals [18,19], it had not previously been demonstrated.
Fig. 3. (Top) Human and rat lumbar vertebral segments are shown for size comparison. (Bottom) In this study, the rat fourth and fifth lumbar vertebrae were fused. Of note, rats have six lumbar vertebrae. The ruler in these figures is 1 cm long.

References


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