Immunological responses to bone soluble proteins in recipients of bone allografts

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Abstract

The significance of an immune response in complications of bone allograft procedures is not well understood. This study evaluates the immunological reaction to bone allografts in either cortical or cancellous form. Serological responses from osteoarthritis (OA) patients and normal individuals to soluble proteins extracted from allografts were assessed using Western blotting and ELISA techniques. A large number of patients expressed antibodies to bone proteins extracted from the washed bone as compared with normal controls. Antibodies were present in patients without the use of bone allografts, indicating that OA patients develop antibodies to bone soluble proteins. However, patients receiving allografts exhibited an increased immune reactivity against multiple bone proteins when compared with non-grafted patients. Protein characterization of the immunoreactive proteins revealed that the majority of antigenic targets were fragments of various collagen molecules. The data suggests that OA patients develop antibodies to bone soluble proteins prior to surgery, and these antibodies increase after revision surgery utilizing bone allografts. These findings support the implications that various collagen molecules as well as their degraded fragments represent potential immunogenic proteins within bone allografts. The removal of these antigenic proteins from the allografts prior to surgery may alleviate this immunological reactivity and improve graft outcome.

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Introduction

Bone tissue was one of the earliest of human transplanted tissues, and today is second only to blood as the most common transplanted material. In the early 1900s, joint allograft transplant procedures occurred with approximately 50% long-term success rate [21]. Despite this history, the literature concerning transplantation immunology of bone remains subdued and ambiguous compared with the enormous body of information published on soft tissue transplantation. Bone does appear to enjoy a considerable measure of transplantation tolerance, and tissue matching of bone between donors and recipients is currently considered as unnecessary. Nevertheless, it is recognized that immunological reactions to bone allografts do occur in both patients and experimental animal models.

Currently, several pre-operative procedures are used to reduce the antigenicity of bone allografts [13,17, 31,29]. The most common preparative procedure is allograft freezing and thawing, which serves to render passenger cells within the graft non-viable. However, while dead cells are less provocative of a strong immunological response than live cells, bone allografting represents a
substantial introduction of foreign proteins into the recipient. Generally, the failure rate for frozen allografts has been in the order of 11–20%, which have been attributed to a combination of factors including rejection, infection, fracture, non-union [27]. Bone allograft rejection by the host immune system has been considered to be a cellular response, however the humoral component may play a role. One researcher suggests that the degree of the host response to an allograft may be linked to the antigen concentration and total dose [5]. Several studies have investigated immune responses to bone allografts, but despite intensive work, in both animal and humans, the significance of the immune response on the fate of bone allografts remains controversial. We hypothesize that other bone antigens besides the MHC antigens expressed on cells may be relevant in the immunological reaction to allografts. Immunological reactions to bone extracted proteins in allograft recipients have been previous reported, but the specificity of these responses has not been evaluated [18,33,15]. Some researches have studied the effects of allograft pre-treatment, particularly freezing, upon bone antigens [2,32,1,3]. Frozen and freeze dried allografts have been shown to remodel and survive in functional form, despite the demonstration in vivo of immune response to these types of grafts [20,6]. While there is little published evidence in patients beyond this observation, several animal studies concur with the concept of adverse reactions to bone antigens during allografting [30,14,37]. One study investigated the healing rates of auto and allograft bone in vivo. They determined that new bone formation started earlier in autografts than in both allografts and syngeneic grafts. Furthermore, antigen-mismatched allografts exhibited retarded formation of new bone throughout the union process [37]. Others have considered the immunologic response against defatted frozen bone allografts, and demonstrated that defatting increased the rate of bone formation rate [35,34]. This data suggests that the increased bone formation rate in defatted allografts might be caused by the removal of allograft antigens, possibly specific cell surface antigens.

One factor that is rarely considered during the evaluation of the allograft outcome is the immunological status of the patient due to the underlying connective tissue disease. Many patients that are undergoing orthopaedic procedures have pathological conditions related to OA. This disease is characterized by the progressive destruction of articular cartilage and the underlying bone. While the immunological aspects of OA are infrequently considered as central to the pathology of the arthritis, there is now evidence of immune reactivity in this disease [24,28,38]. Minor antigenic variations in connective tissue components occurs between individuals; therefore it is conceivable that certain allograft recipients may respond to foreign or autologous bone antigens due to their arthritic condition. To date, the cumulative effects of an immunological response to MHC antigens expressed upon the allograft and a reaction to connective tissue antigens present in the foreign bone have not been examined in the outcome of allograft procedures. It was our intention to evaluate the responses to these entities, using established immunological techniques. The objective of this study was to determine the significance of immunological responses to non-major histocompatibility (MHC) bone antigens in the outcome of allografts in OA patients, and characterize immunoreactive proteins within bone that might influence the long-term outcome of bone allografts.

Methods

Patients

The study was conducted in patients (n = 121) with a diagnosis of OA of the hip and/or knee presenting with a failed hip or knee arthroplasty and considered as candidates for a revision total knee arthroplasty (TKA) or total hip arthroplasty (THA). Patients were classified into three groups according to their surgical management: Subjects having no bone allograft used during the revision procedure served as controls (Group 1, n = 43), while the allograft groups were divided into cortical (Group 2, n = 32) and cancellous grafts (Group 3, n = 46). There were 38 men and 57 women ranging in age from 41 to 87 years (average age = 68 ± 10.6) with no statistically significant difference in age between groups. All patients were cleared by a physician as being medical stable. Patients that received allografts during primary arthroplasty were excluded from the study, as they could already be sensitized. Other exclusions included patients with a history of rheumatoid arthritis, avascular necrosis, and traumatic injury to the hip and knee. Patients were also restricted to first line medical therapy for their arthritic conditions (non-steroidal anti-inflammatory agents only). Venous blood was collected for analysis in plain tubes and tubes containing preservative-free heparin. Blood collection occurred intra-operatively and at the six month post operative check. All procedures were approved by the Wayne State University Human Investigation Committee.

Saline wash pre-treatment of bone allografts

Allograft bone used in these procedures was obtained from three commercial sources: The Musculoskeletal Transplant Foundation, the Georgia Tissue Bank, and the tissue bank at the University of Miami. Allografts were delivered frozen, and stored at −80 °C until used. The bone was thawed in warm normal saline. Cortical grafts were cut to shape, all visible cellular material removed, and then the grafts were washed in warm normal saline until no fat or protein was visible in the effluent. Cancellous grafts were prepared intra-operatively using the Ling-Slooff technique [16]. Briefly, the bone is ground in a Tracer bone mill until the particulate granules are approximately 0.5 cm². The ground bone is then washed in warm saline (40 °C) until the effluent was clear of visible fat and protein. In general this required five to seven wash cycles. The bone was then used for allografting.

Preparation of bone soluble proteins (BSP)

The bone saline wash effluent was pooled, and refrigerated at 4 °C overnight to allow for the solidification of lipids. Five hundred milliliters of clarified solution was removed and placed into concentrators (Amicon, Inc., Beverly, MA) to concentrate proteins above a molecular weight of 10 kDa. Centriprep were centrifuged at 2000g for one hour, and the protein concentration in the BSP extract was determined.
using the BCA protein assay (Pierce; Rockford, IL) in accordance with the manufacturer's instructions. A bovine sera albumin protein (BSA) standard of known concentration was used for calibration.

Gel Electrophoretic assessment of BSP

SDS-PAGE was performed using a 7.5% polyacrylamide separating gel and a 4% stacking gel. After electrophoresis, the gel (with lanes containing both the BSP solution and a molecular weight ladder) was stained with Coomassie Blue. The number and molecular weight of the bands in the BSP extract was determined by comparison against a standard protein ladder by image analysis. Gels were dried and stored as permanent records.

Western blot analysis of anti-BSP antibodies

IgG was extracted from the BSP solution by passage through a Protein G HiTrap column (Sigma Chemical Co.; St Louis, MO), and immunoglobulin-free eluent solution used for Western blot analysis. SDS-PAGE gels, prepared as above, were electrotransferred to nitrocellulose membranes. Membranes were cut into strips corresponding to the electrophoretic gel lanes, blocked in PBT (50 mM Tris-HCl pH 7.4, 0.2% Triton X-100, 1 mM EDTA, 10 mM NaN3, 0.15 M NaCl) and 4% BSA (Fisher Scientific; Fair Lawn, NJ). Membrane strips were incubated with patient sera at a dilution of 1:500. After primary incubation, the membrane strips were washed 3x for 5 min each in GB (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 2 mM K-EDTA, 0.5% Triton X-100, 0.1% SDS). The strips were then incubated with anti-human Ig antibody coupled with horseradish peroxidase (Amersham Life Science; Piscataway, NJ) at a dilution of 1:500. After incubation the membrane strips were washed 3x for 5 min in GB, removed, incubated in ECL Western blotting detection reagents (Amersham Life Science; Piscataway, NJ) and exposed to high performance chemiluminescence films. The number and molecular weight of the bands on the films was determined using image analysis (AlphaInnotech, San Leandro, CA).

Enzyme linked immunosorption assay (ELISA)

ELISA was used to evaluate serum antibodies that specifically react with native and/or denatured type I collagen. Briefly, type I collagen was dissolved in 0.1 M acetic acid and 96 well plates coated with the protein solution at a concentration of 3 µg/well. Plates were washed with Phosphate Saline Buffer (PBS)/Tween, and blocked with 5% milk/PBS. Patient sera samples were diluted at 1:500 in milk/PBS, incubated then washed with PBS/Tween. Plates were incubated with goat anti-human IgG was extracted from the BSP solution by passage through a Protein G HiTrap column (Sigma Chemical Co.; St Louis, MO), and immunoglobulin-free eluent solution used for Western blot analysis. SDS-PAGE gels, prepared as above, were electrotransferred to nitrocellulose membranes. Membranes were cut into strips corresponding to the electrophoretic gel lanes, blocked in PBT (50 mM Tris-HCl pH 7.4, 0.2% Triton X-100, 1 mM EDTA, 10 mM NaN3, 0.15 M NaCl) and 4% BSA (Fisher Scientific; Fair Lawn, NJ). Membrane strips were incubated with patient sera at a dilution of 1:500. After primary incubation, the membrane strips were washed 3x for 5 min each in GB (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 2 mM K-EDTA, 0.5% Triton X-100, 0.1% SDS). The strips were then incubated with anti-human Ig antibody coupled with horseradish peroxidase (Amersham Life Science; Piscataway, NJ) at a dilution of 1:500. After incubation the membrane strips were washed 3x for 5 min in GB, removed, incubated in ECL Western blotting detection reagents (Amersham Life Science; Piscataway, NJ) and exposed to high performance chemiluminescence films. The number and molecular weight of the bands on the films was determined using image analysis (AlphaInnotech, San Leandro, CA).

Identification of protein constituents of the BSP extract

Standard bone proteins (osteocalcin, osteonectin, native type I and II collagen) and the corresponding antibodies to these proteins were obtained from commercial sources (Calbiochem-Novabiochem Corporation; La Jolla, CA) and through collaborative arrangements. Western blots were conducted to evaluate antibody responses in patient sera against the standard bone proteins. SDS-PAGE and electrotransfer were performed and the blots were exposed to mouse antisera specific for standard proteins. Membranes were washed and incubated with anti-mouse Ig antibody coupled with horseradish peroxidase (Sigma Chemical Co.; St Louis, MO). After membranes were washed, blot developed and analyzed as described above. Immunoreactive components of the BSP extract were purified, isolated and N-terminus amino acid sequenced by the Protein/Nucleic Acid Facility of the Michigan State University. The amino acid sequences were submitted to the protein databases, in order to identify probable candidate proteins. Subsequently, these proteins and their appropriate specific antibodies were used to confirm the identity of the immunoreactive components of the BSP extract.

Statistics

Statistical analyses were conducted using SPSS® (SPSS Inc., Chicago, IL). Differences among groups were evaluated using Chi-squared tests, with the Yates correction as necessary. For differences between or among groups, the analysis of variance (ANOVA) was performed with post hoc pairwise testing, when necessary, using the Scheffe test. An alpha level of 0.05 was selected for significance for all of the statistical tests.

Results

Immunological reactivity to BSP

Antibody responses to BSP were detected in the majority of OA patients. These responses increased subsequent to revision arthroplasty using bone allografts. Evaluation of the immunoblots revealed a high number of reactive sera, with 66 of the 120 allograft recipients (55%) generating one or more reactive bands specific for BSP. In contrast, only 2 of 12 control sera (16%) were reactive on the Western blots. Using the Chi-squared test, this reveals a highly significant elevation (p < 0.001) of anti-BSP antibodies in the allograft recipients compared to the controls. When examining the non-grafted patients versus the cortical and cancellous grafted individuals, we found that 38% of the non-grafted OA patients presented with antibodies to at least one of the BSP, as compared to 59% of the cortical and 67% of the cancellous grafted patients (Fig. 1). This data reached significance (p < 0.006) when comparing the cancellous-grafted versus the non-grafted group.

Identification of antigenic determinants in BSP solutions

The molecular weights of the most common immunoreactive proteins were 30 kD (36 patients), 40 kD (29...
patients) and 65 kD (11 patients). The distribution of these proteins is shown in Table 1. We considered that the major protein in bone, type I collagen (MW = 110 kD) might represent a key antigen in the immune response and pathogenesis of OA. We therefore used ELISA to determine whether OA patient sera contained antibodies to native type I collagen. The results were negative for both the patients and the normal individuals. However, type I collagen in its native form enjoys a high level of immunological tolerance. We therefore considered that immune reactivity might be directed against damaged or denatured collagen in OA. Type I collagen was denatured by heating to 60 °C for 10 min followed by rapid cooling, then the ELISA was repeated with this denatured protein. This assay revealed that 66% of patients had antibodies to collagen in the denatured form (Fig. 2) in the absence of reactivity against native type I collagen. Examination of other proteins commonly found in bone led to the consideration of osteonectin as possible candidate for the 30 kD immunoreactive protein. Recombinant human osteonectin was used to generate antibodies in mice, which were confirmed as specific by the ELISA technique. In comparative Western blot assays, osteonectin was not proved to be an immunoreactive protein of the bone wash extract in the patient sera tested. However, this findings should not be considered conclusive, since a reaction to osteonectin might be directed against a denatured or damaged form as seen for type I collagen.

**Protein sequencing of antigenic targets**

Immunoreactive proteins were submitted to sequence analysis for identification, with six proteins (molecular weights of 140, 65, 40, 30, 15 and 7 kD) being investigated. However, through the preparative high performance liquid chromatography (HPLC) purification process of the 140 kD molecular weight protein was found to be a 70 kD dimer. The 65 kD protein was identified as human serum albumin (HSA). Following this result, we assayed all allograft patient sera, as well as normal sera, for reactivity to HSA using the ELISA technique. The results of this test indicated that none of the sera contained antibodies specific for native HSA, which again raises the possibility of immune responses directed against a denatured protein. The remaining sequences compared against the protein database using the search program FASTA (www.fasta.bioch.virginia.edu). The results show the location of our queries within the total amino acid sequence, as well as giving the percent of amino acid matches. We compared these results with a program that uses the European human protein database (www.ebi.ac.uk). Table 2 shows the results from the database searches, with the amino acid matching score (100 representing a complete identity score). The search reveals three potential antigenic targets; namely Pulmonary surfactant-associated protein D precursor, hemoglobin and fragments of the type I collagen molecule.

### Table 1

<table>
<thead>
<tr>
<th>Distribution of immunoreactive proteins within OA patients</th>
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<tr>
<td>MW &gt; 210</td>
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<tr>
<td>----------------------------------------------------------</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Cortical bone recipients</td>
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<td>Cancellous bone recipients</td>
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**Fig. 2.** The percent of antibodies to denatured type I collagen established by the ELISA technique. This assay revealed that 66% of patients had some level of antibodies to collagen in the denatured form in the absence of reactivity against native type I collagen.

### Table 2

<table>
<thead>
<tr>
<th>Protein (kD)</th>
<th>Match (Score)</th>
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<tr>
<td>70 Pulmonary surfactant-associated protein D precursor (86.7)</td>
<td></td>
</tr>
<tr>
<td>Collagen alpha 2 (IV) chain precursor (76.7)</td>
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</tr>
<tr>
<td>Collagen alpha 1 (VI) chain precursor (72.2)</td>
<td></td>
</tr>
<tr>
<td>Collagen alpha 1 (II) chain precursor (70)</td>
<td></td>
</tr>
<tr>
<td>40 Collagen alpha 1 (IX) chain precursor (69.6)</td>
<td></td>
</tr>
<tr>
<td>Collagen alpha 2 (VIII) chain fragment (62.7)</td>
<td></td>
</tr>
<tr>
<td>Collagen alpha 1 (XI) chain precursor (62.7)</td>
<td></td>
</tr>
<tr>
<td>30 Hemoglobin beta chain (93.3)</td>
<td></td>
</tr>
<tr>
<td>15 Hemoglobin alpha chain (100)</td>
<td></td>
</tr>
<tr>
<td>~7 Collagen alpha 2 (I) chain precursor (67.9)</td>
<td></td>
</tr>
<tr>
<td>Collagen alpha 3 (IV) chain precursor (67.9)</td>
<td></td>
</tr>
<tr>
<td>Collagen alpha 2 (IV) chain precursor (65.5)</td>
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Discussion

While bone is commonly believed to be immunologically privileged as a transplantation tissue, it is nevertheless recognized as antigenic in bone graft recipients, and several pre-operative procedures have been used to reduce the antigenicity of bone allografts. While allograft freezing renders passenger cells within the graft non-viable, the graft still represents a substantial introduction of a foreign protein into the recipient. Another pre-operative procedure being utilized to decrease bone allograft antigenicity is warm saline washing. We evaluated immunological responses in our patient population in the light of evidence that saline wash pre-treatment of bone allografts appears to clinically improve graft outcomes. The proteins collected from bone allograft wash solutions were used to examine the humoral immune responses from patients with OA who underwent revision arthroplasty. The data support the hypothesis that a large number of OA patients have antibodies to several proteins present in bone wash solutions. The first protein identified was HSA, the major protein in blood plasma. This was a surprising result, since it is rare that individuals produce an autoantibody to this abundant serum protein. However, anti-albumin antibodies have been described in patients with diabetes mellitus, and liver diseases such as viral hepatitis [26,4,36,8] and liver cirrhosis [22]. Since whole bone, including bone marrow, was ground and used for allografting, it is inevitable that HSA would be present in the protein extract. The medical records of the patients with antibodies to HSA revealed many concomitant conditions, including some of those associated with anti-HSA reactions. However, there was no obvious link between HSA-reactive orthopaedic patients and any one specific medical condition that would explain this immune reactivity. Two possible explanations can be considered; patients may recognize denatured HSA within the donor tissue as foreign, and develop a response. Alternatively, HSA may act as a carrier protein or adjuvant in the response to the allograft, since many studies have demonstrated that HSA has been linked to adjuvant activity and can stimulate an immune response against other antigens [10,19,23]. It is possible that HSA is binding to other proteins in graft, resulting in conformational changes and the recognition of foreign antigens.

Protein sequencing of the immunoreactive antigens within the bone wash identified several novel targets for consideration. One unexpected finding was the identification of the hemoglobin components. It is evident that hemoglobin is present within the bone wash solution, since bone marrow represents a major source for this protein. When red blood cells are lysed, hemoglobin is released within the circulation. Since free hemoglobin is unstable, it usually binds to other proteins, such as haptoglobin and HSA. Although autoantibodies against this protein are rare in human disease conditions, it is possible to generate antibodies to human hemoglobin in labora-

tory animals. A recent study reports that anti-hemoglobin antibodies may bind to the red blood cell surface [12].

The identification of elements of the type I collagen molecule as antigenic targets in bone grafts appears both reasonable and significant. The identification of anti-collagen antibodies within individuals with arthritis is not new, as a number of publications have reported upon this immune reactivity [28,11,9,7]. One hypothesis concerning the progression of osteoarthritis suggests that the 'wear and tear' of cartilage and bone within the joints may lead to an immune response against degraded collagenous proteins. The collagen superfamily of proteins includes more than 20 different collagen types with at least 38 individual polypeptide chains and more than 15 additional proteins that have collagen-like domains [25]. Minor antigenic variations in connective tissue components occur between individuals; therefore, it is conceivable that certain allograft recipients may respond to foreign or autologous bone antigens due to their arthritic condition. Since OA is related to the degradation of cartilage and bone, denatured forms of proteins may present as foreign to an active immune system. Thus, antibodies to denatured collagen fragments may occur within the orthopaedic patient population. The sequencing results in this study identified possible antigen candidates as types I, II, VIII, VI, IX, and XI collagen, suggesting that many bone proteins hold the potential to serve as antigens following grafting. Several studies have linked antibodies to collagen molecules with pathological conditions, including a report that 50% of patients with rheumatic diseases develop antibodies to type IX collagen [7]. The same study also found the frequent presence of antibodies to type IX collagen. A recent study has reported the existence of type IX collagen immunoreactive peptides within arthritic patients as compared to normal individuals [38]. These findings support the implications from our protein sequencing data that the various collagen molecules as well as their degraded fragments represent potential immunogenic proteins within bone allografts.

Acknowledgement

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References


