EXPRESSION OF PROTEOGLYCAN EPITOPES IN ARTICULAR CARTILAGE REPAIR TISSUE

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ABSTRACT

To determine if expression of specific proteoglycan epitopes distinguishes articular cartilage repair tissue from normal articular cartilage, we used seven monoclonal antibodies to examine normal articular cartilage and cartilage repair tissue from osteochondral defects 3.2 mm in diameter and 4.0 mm deep in the medial femoral condyles of 27 New Zealand white rabbits and seven cynomolgus monkeys. Following creation of the osteochondral defects, one limb of each animal was treated with cast immobilization while the other limb was treated with passive motion for two weeks. Rabbit knees were examined at eight (13 animals, 26 knees) and 36 weeks (14 animals, 28 knees) and monkey knees at eight weeks (seven animals, 14 knees) following surgery. Staining for six of the antibodies did not differ between repair cartilage and normal articular cartilage, but an antibody that recognizes atypical glycosaminoglycan structures in developing tissues (MAb 7D4) consistently distinguished repair cartilage from normal cartilage in rabbits and monkeys. Repair tissue consisting of hyaline toluidine blue-staining matrix containing chondrocytic cells uniformly showed strong 7D4 staining. In contrast, normal articular cartilage and fibrous repair tissue showed inconsistent weak 7D4 staining. At eight weeks following surgery, rabbit cartilage repair tissue stained more intensely for 7D4 than monkey cartilage repair tissue; in rabbits, cartilage repair tissue stained more intensely for 7D4 at eight weeks than at 36 weeks following surgery. Repair tissue staining for 7D4 did not differ between osteochondral defects treated with passive motion and those treated with immobilization in rabbits and monkeys. These results indicate that expression of a high level of proteoglycan epitope 7D4 distinguishes hyaline articular cartilage repair tissue from normal articular cartilage and fibrous cartilage repair tissue in the early stages of osteochondral healing, and that as hyaline articular cartilage repair tissue matures expression of 7D4 decreases. The ability to characterize repair cartilage proteoglycans with monoclonal antibodies may aid in the evaluation of the quality and maturity of cartilage repair tissue and thereby facilitate improvements in procedures for resurfacing joints.

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Supported by NIH Grants AR 38733 and 40364

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INTRODUCTION

The pain and decreased mobility caused by loss of articular cartilage have led investigators to seek methods of repairing or regenerating articular surfaces[2,4]. Articular cartilage does not regenerate in experimental defects limited to articular cartilage[1,13,17], but in defects that penetrate subchondral bone, a variable repair response occurs, often resulting in a mixture of cartilaginous and fibrous tissue that in some instances includes new hyaline-like cartilage[1-4,6,12]. In an attempt to induce articular cartilage regeneration in humans, surgeons have abraded subchondral bone or made multiple small

holes into bone[2,5,15,16,19]. Some patients notice a decrease in symptoms, but others notice little or no benefit[2,16]. Overall, the clinical results generally deteriorate with time and examination of a few specimens has demonstrated that the repair tissue usually consists of a mixture of fibrocartilage and hyaline cartilage[2,4,16]. Other methods of repairing or regenerating articular surfaces include osteotomies and use of periosteal and perichondrial grafts, chondrocyte transplants and artificial matrices. The results of these procedures also appear to vary among patients[2,4].

Either insufficient synthesis of proteoglycans or differences between the proteoglycans synthesized in cartilage repair tissue and normal articular cartilage may help explain this variability and the deterioration of most articular cartilage repair tissue with time. Mitchell and Shephard attempted to stimulate regeneration of an articular surface in rabbit knees with multiple small drill holes into subchondral bone[18]. Initially, the new joint surface had the appearance of hyaline articular cartilage; however, after eight months it had deteriorated and examination showed that it consisted of dense collagenous tissue. Furukawa and colleagues also found that articular cartilage repair tissue changed with time. They reported that the change from a hyaline to a fibrocartilaginous appearance was not due to loss of type II collagen[12]. At one year, type II collagen was the predominant collagen in cartilage repair tissue, and its concentration had increased compared with earlier stages of cartilage repair. They suggested that the deterioration of the repair tissue resulted from a loss of proteoglycans. Other researchers have also noted apparent loss of proteoglycans and degeneration of cartilage repair tissue after six months[5,6,21]. Despite the general pattern of cartilage repair tissue degeneration with time, newly formed articular surfaces in some patients appear to perform well for years[2,5]. In some animal osteochondral defects, a new cartilage surface forms that closely resembles normal articular cartilage in gross and microscopic appearance, proteoglycan staining properties, and type II collagen concentration at more than six months after creation of the defect[6]. Thus, methods of evaluating cartilage proteoglycans could be helpful in defining the differences between cartilage repair tissue and normal articular cartilage and in explaining why some cartilage repair tissue matures so that it resembles normal articular cartilage.

Monoclonal antibodies, generated against various epitopes in chondroitin sulfate glycosaminoglycans[8-11, 22], have demonstrated subtle changes in the sulfation patterns of cartilage glycosaminoglycans during growth and development and in joint diseases[8-11, 22]. The primary purpose of this study was to use these antibodies to determine if expression of different proteoglycan epitopes distinguishes articular cartilage repair tissue from normal articular cartilage and other types of repair tissue. A secondary purpose was to determine if passive motion treatment of osteochondral defects[20] alters the expression of proteoglycan epitopes in cartilage repair tissue.

MATERIALS AND METHODS

The 27 New Zealand white rabbits (six to eight months old) and seven adult wild-captured cynomolgus monkeys included in this study were treated in accordance with N.I.H. animal care guidelines. After induction of general anesthesia, the medial femoral condyles of both knees of each animal (27 rabbits, 54 knees, and seven monkeys, 14 knees) were exposed by medial parapatellar incisions. A drill hole 3.2 mm in diameter and 4.0 mm in depth was placed in each posterior medial femoral condyle. One leg was placed in a long leg cast, and the other leg was placed in a passive motion apparatus for two weeks after surgery The machine continuously performed one cycle every 40 seconds in rabbits and for 16 hours followed by eight hours of rest in monkeys. Subsequently, animals were allowed to move freely within their cages. Thirteen rabbits and all seven monkeys were killed by lethal injection eight weeks following surgery. Fourteen rabbits were killed 36 weeks following surgery by the same method. The distal femurs were fixed in 10% neutral buffered formalin, decalcified in 10% EDTA, embedded in paraffin, and sectioned to seven micron thickness.

One central section from each defect was stained with toluidine blue and rated for the quantity of hyaline cartilage repair tissue in the chondral defect relative to the total size of the defect according to the following scale: 0 - no hyaline tissue, 1 - less than one third hyaline tissue, 2 - one third to two thirds hyaline tissue, and 3 - more than two thirds hyaline tissue. Hyaline cartilage repair tissue was defined as tissue consisting of a hyaline toluidine blue-staining matrix containing spherical cells with the morphologic features of chondrocytes.

Twenty to 30 sections from each defect were prepared for immunohistochemical staining. They were deparaffinized in xylene and hydrated successively in 100%, 90%, 70%, 35% ethanol, distilled water, and Tris-saline buffer (100 mM NaCl, 50 mM Tris pH 7.4). Some sections (depending on the antibody used) were treated with chondroitinase ABC lyase (0.1 unit/ml in 1% bovine serum albumin (BSA), 100 mM Tris acetate pH 7.9). Slides were placed in 0.25% H_2O_2 in methanol for 20 minutes, washed with Tris-saline buffer, and loaded into a Shandon Sequenza apparatus. They were blocked with 20% sheep serum for 20 minutes and washed with

Tris-saline buffer. Slides were successively incubated with the following reagents with intervening Tris-saline buffer washes: first antibody, diluted 1:100 in Tris-saline buffer with 1% BSA, for 30 minutes; second antibody, biotinylated goat anti-mouse polyvalent immunoglobulin (Sigma), diluted 1:100 in Tris-saline buffer with 1% BSA for 30 minutes; peroxidase conjugated streptavidin (Sigma) 2.5 mg/ml in Tris-saline buffer with 1% BSA for 30 minutes; and finally, DAB solution, 0.05% 3, 3'-diaminobenzidine tetrahydrochloride, 0.15% H_2O_2 in Tris-saline buffer, for 15 minutes. Sections were washed with Tris-saline buffer and distilled water, dehydrated, and mounted.

Sections were rated for intensity of immunohistochemical staining by a panel of monoclonal antibodies directed against a variety of glycosaminoglycan structures, and one antibody directed against link protein (Table 1). Each antibody was used in two or more sections from each defect. Ratings of the intensity of immunohistochemical staining were performed independently of the ratings of the quantity of hyaline cartilage repair tissue. The overall intensity of the color reaction in the repair tissue was compared to the staining in normal cartilage in the same specimen and rated according to the following scale: 0 - less staining, 1 - equal staining, and 2 - greater staining. A numeric value for staining intensity was not scored because of the variation in staining of normal articular cartilage between animals.

The Mann-Whitney test was used to compare two groups with non-parametric ratings (quantity of hyaline cartilage repair tissue and staining intensity ratings).

Table 1. Monoclonal Antibodies

Monoclonal Antibody	Class	Location of Epitope
7D4	IgM	Native chondroitin sulfate
4C3	IgM	Native chondroitin sulfate
6C3	IgM	Native chondroitin sulfate
5D4	IgG	Keratan sulfate penta and heptasaccharides
3B3	IgM	Non-reducing terminal delta-unsaturated or saturated disaccharides of chondroitin-6-sulfate produced by chondroitinase or hyaluronidase
2B6	IgG	Non-reducing terminal delta-unsaturated disaccharides of chondroitin-4-sulfate generated by chondroitinases
8A4	IgG	Link proteins from all animal species from shark to human

Spearman's rank correlation coefficient was used to test the relationship between two non-parametric ratings. Statistical significance was defined at the $p < 0.05$ level. No differences were found between cast and passive motion treated osteochondral defects in quantity of hyaline cartilage repair tissue or intensity of immunohistochemical staining. Therefore, these groups were combined for analysis of the relationship between quantity of hyaline cartilage repair tissue and immunohistochemical staining (14 osteochondral defects in monkey knees eight weeks following surgery, 26 osteochondral defects in rabbit knees eight weeks following surgery and 28 osteochondral defects in rabbit knees 36 weeks following surgery).

RESULTS

The quantity of hyaline cartilage repair tissue varied among rabbits and monkeys within the same experimental groups (Figures 1A and 1B, and Figures 1C and 1D). Despite the variability within groups, a significant difference existed between rabbits and monkeys. At 36 weeks, there continued to be a range of repair responses in rabbit osteochondral defects from fibrous to cartilaginous tissue and the repair tissue in some defects had developed degenerative changes (Figures 1E and 1F). The quantity of hyaline cartilage repair tissue did not differ between defects treated with cast immobilization and passive motion ($p = 0.38$ for monkeys, $p = 0.11$ for rabbits at eight weeks, and $p = 0.64$ for rabbits at 36 weeks) or between those rabbits studied eight and 36 weeks following surgery ($p = 0.84$). The quantity of hyaline cartilage repair tissue was greater in rabbits than monkeys at eight weeks after surgery ($p < 0.001$).

One of the seven antibodies studied, 7D4, produced strikingly consistent differences in staining between normal articular cartilage and repair cartilage (Figure 2). This antibody stained normal articular cartilage faintly in both rabbits and monkeys, usually in the superficial zone (Figures 2A and 2C). At eight weeks, both monkey and rabbit hyaline cartilage repair tissue showed diffuse and greater staining with 7D4 than normal cartilage, although the intensity of staining in monkey hyaline repair cartilage was less than in rabbits (Figures 2B and 2D). At 36 weeks, the proportion of rabbit defects containing repair tissue that stained more intensely for 7D4 than normal articular cartilage had decreased ($p < 0.001$). Defects filled with fibrous tissue, as shown in Figures 1A and 1B, stained weakly with 7D4, and strong positive correlations existed between scores for 7D4 staining and scores for quantity of hyaline cartilage repair tissue in both monkeys and rabbits (Spearman's rank correlation coefficient $rs = 0.84$ for monkeys at eight weeks, $rs = 0.82$ for rabbits at eight

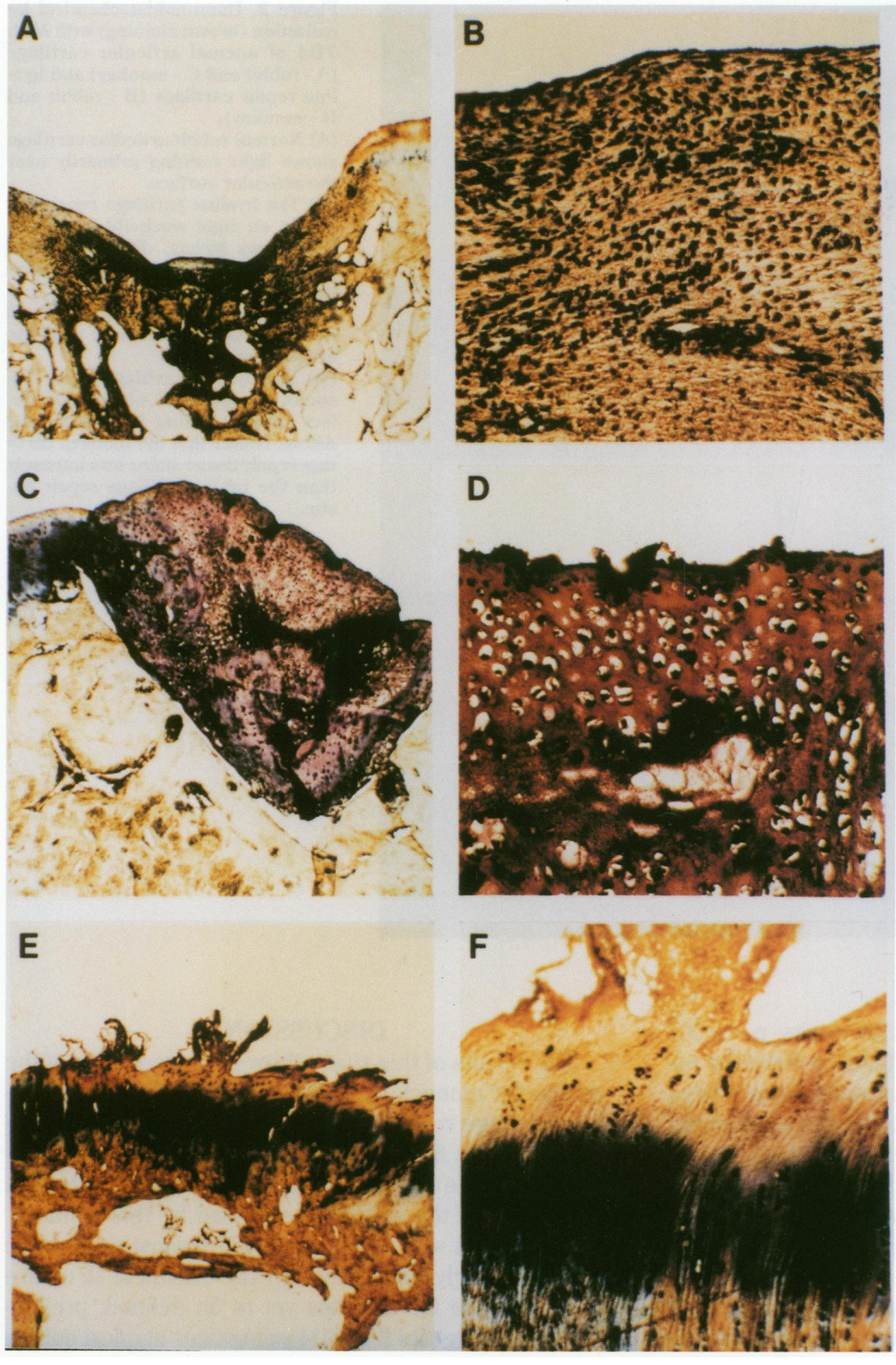

Figure 1. Light micrographs showing toluidine-blue staining of 3.2 mm diameter osteochondral defects in the medial femoral condyles of three rabbits. A, B, C and D are from rabbits at eight weeks after surgery. E and F are from a rabbit at 36 weeks after surgery. Panels B, D and F are high power magnifications of A, C and E, respectively.
(A) The repair rating was 0 in this specimen (no hyaline-like cartilage was visible).
(B) The higher power magnification of the section in A shows fibrous tissue.
(C) The repair rating was 3 in this defect (primarily hyaline-like cartilage).
(D) High power magnification of the section in C shows chondrocytes in lacunae and metachromatic-staining of the matrix.
(E) In this specimen, there is marked surface fibrillation and loss of surface staining for toluidine blue.
(F) High power magnification of the section in E.

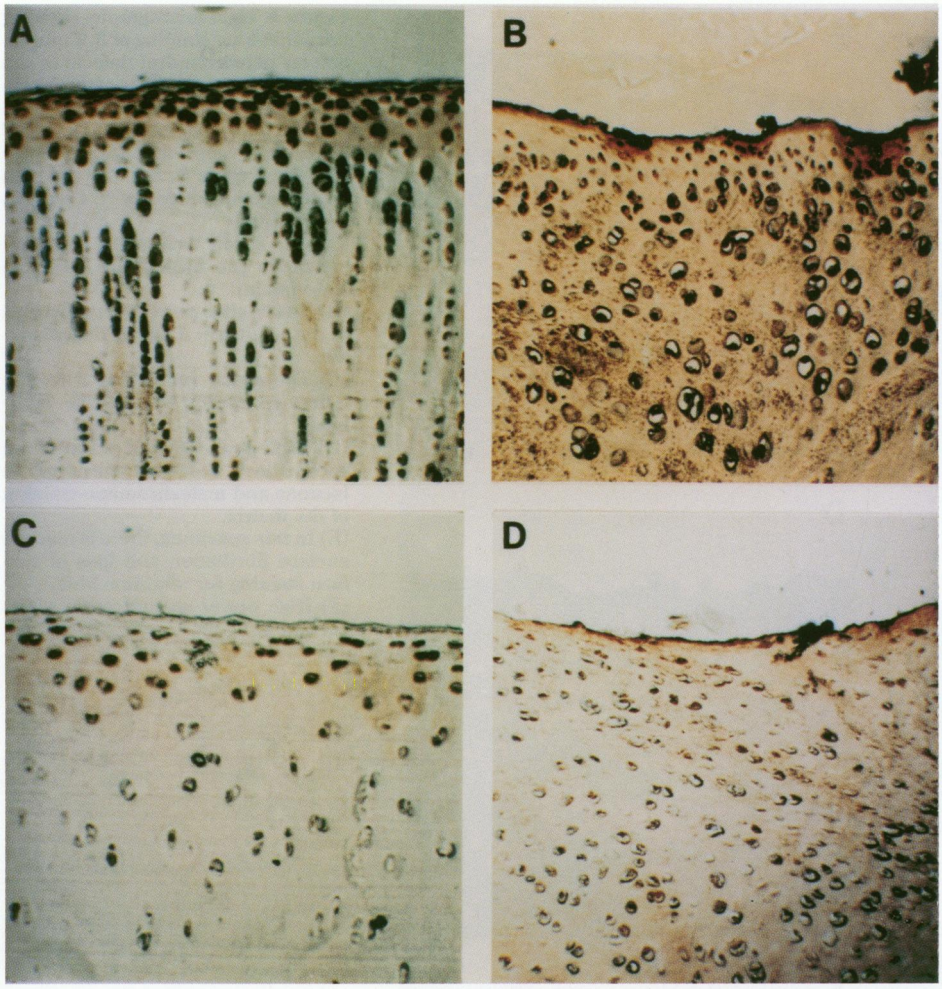

Figure 2. Immunohistochemical localization (brown staining) with MAb 7D4 of normal articular cartilage (A - rabbit and C - monkey) and hyaline repair cartilage (B - rabbit and D - monkey).
(A) Normal rabbit articular cartilage shows light staining primarily near the articular surface.
(B) The hyaline cartilage repair tissue in an eight week-old rabbit defect shows strong, diffuse staining throughout the newly formed cartilage.
(C) Normal monkey cartilage shows minimal staining primarily near the articular surface.
(D) The hyaline cartilage repair tissue in an eight week-old monkey defect shows staining throughout the defect. Notice that the monkey cartilage repair tissue stains less intensely than the rabbit cartilage repair tissue.

weeks, and rs = 0.72 for rabbits at 36 weeks, $p < 0.001$ for all groups). There was no difference between osteochondral defects treated with a cast and those treated with passive motion with respect to scores for staining with 7D4. The other six antibodies showed equivalent staining in normal cartilage and hyaline cartilage repair tissue, and control sections with no first antibody demonstrated no artifactual staining with the second antibody.

To determine whether the increased staining with 7D4 could be explained by an increased concentration of proteoglycans as demonstrated by toluidine blue staining, the samples with increased 7D4 staining in repair cartilage were examined for the intensity of toluidine blue staining relative to normal cartilage. In rabbits, toluidine blue staining was stronger in normal cartilage compared to repair cartilage ($p < 0.001$). In monkeys, there was equivalent toluidine blue staining in repair and normal cartilage ($p = 0.44$).

DISCUSSION

The results of this study show that expression of the epitope for monoclonal antibody 7D4 distinguishes hyaline cartilage repair tissue from normal articular cartilage and from fibrous repair tissue in rabbits and monkeys. Passive motion treatment did not alter the expression of the epitope recognized by this antibody. Antibody 7D4 was originally raised against embryonic chick proteoglycans. Although the chemical structure of the epitope for 7D4 has yet to be defined, preliminary data suggests that 7D4 recognizes atypical sulfate patterns in chondroitin sulfate glycosaminoglycans which are abundant in developing embryos[23]. The intense staining for 7D4 in hyaline repair cartilage at eight weeks following creation of osteochondral defects suggests that during the process of articular cartilage repair, cells that assume the appearance of chondrocytes[21] synthesize embryonic glycosaminoglycan structures. Replacement of these structures by other glycosaminoglycans may lead to formation of tissue that more

closely resembles mature articular cartilage, but this study was not designed to test this possibility.

At eight weeks following surgery, rabbit osteochondral defects contained more hyaline cartilage repair tissue than monkey osteochondral defects and, thus, stained more intensely for 7D4. These observations indicate that the rabbits produced a more effective cartilage repair response than the monkeys. This may result from differences between the two species, but it may also result from differences in the mean ages of the animals studied. That is, the ability to regenerate articular cartilage may decline with age[7], and the monkeys probably were older than the rabbits. Unfortunately, the age of the monkeys was unknown.

Our study has other important limitations. First, the differences between normal cartilage and repair cartilage in 7D4 staining were consistent and striking, but since evaluation of the intensity of tissue staining could not be precisely measured, more subtle differences in staining of normal and repair cartilage with antibodies other than 7D4 (Table 1) may not have been detected. Second, we used drill holes to induce articular cartilage repair. It is possible that other methods of stimulating cartilage formation, such as perichondrial or periosteal grafts, cell transplants, or use of artificial matrices may produce different results. Finally, the failure to detect differences between defects treated with immobilization and passive motion has several possible explanations. First, our rating systems for quantifying hyaline cartilage repair tissue and immunohistochemical staining may have failed to detect subtle differences, or differences may have been present sooner than eight weeks after creation of the defects. Another possible explanation is that slight motion in the casted limbs may have made the conditions for cartilage repair similar to those of passive motion[14,20].

Despite these limitations, this study shows that novel glycosaminoglycan structures are present on proteoglycans early in the course of articular cartilage repair following creation of osteochondral defects, and that the presence of a relatively high concentration of these structures distinguishes hyaline cartilage repair tissue from fibrous repair tissue and from normal articular cartilage. In addition, it appears that hyaline cartilage formation in osteochondral defects involves cells that have assumed a chondrocytic phenotype[21] and may require expression of epitopes also expressed during embryonic development, and that with maturation of cartilage repair tissue expression of these epitopes decreases. Further studies are needed to determine if repair tissue that initially expresses these epitopes produces better long term restoration of an articular surface and if cartilage repair tissue produced by other methods including osteotomies, periosteal and perichondrial grafts and cell transplants also expresses these epitopes.

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