On both the paralyzed and unaffected sides, lower extremity muscle mass during the first measurement was lower than that during the second measurement, and the difference between the two measurements was 469 g (SD : 179) on the paralyzed side and 320 g (SD : 178) on the unaffected side. In Case B, upper extremity muscle mass during the second measurement was higher than that during the first measurement, but in the other two patients, upper extremity muscle mass during the first measurement was higher than that during the second measurement.

2. Predictors for low lower extremity muscle mass

Table 2 compares the factors for low muscle mass in

Table 2. Profile of three patients

	Case A	Case B	Case C
Age and gender	80 year-old man	77 year-old man	73 year-old woman
Disease	Cerebral bleeding	Cerebral infarction	Cerebral bleeding
Main therapy	Precise microinjection of hypotensive agent for 5 days	Precise microinjection of hypotensive agent for 5 days	Precise microinjection of hypotensive agent for 9 days
Complications	Hypertension	Hypertension and DM	Hypertension
Consciousness level GCS :			
first test	E4M6V3	E4M6V1	E3M5V3
Second test	E4M6V3	E4M6V1	E3M6V3
Paralyzed side	Right	Right	Right
Paralyzed-side movement			
Brunnstrom stage Lower extremity	I	II	I
Upper extremity	I	II	I
Physical activity (within 10 days of onset)	Remained in bed Getting up : 30−45°	Remained in bed Getting up : 45−90°	Remained in bed Getting up : 45−90°
Swallowing disorder	Yes	Yes	Yes
Aphasia	Yes	Yes	Yes
Communication	Communicate using gestures	Communicate only through eye contacts	Communicate using gestures
Other relevant symptoms	Diarrhea, restlessness, and physical restraint for restlessness	Diarrhea, fever, and passive movements of upper extremities	Diarrhea, and able to raise the unaffected knee and elevate the hips
Nutritional state total protein			
(g/dl) on admission	7.3	7.8	7.2
First test	5.4	5.6	6.4
Second test	5.7	6.8	6.5
Start of oral intake (after admission)	4 days	8 days	3 days
Food intake	Tubal feeding	Tubal feeding	Tubal feeding
Duration of drip infusion (days)	15	17	8
Discharge from SCU after admission	Sixth day	Sixth day	Tenth day
Body weight (kg) First test	45.9	58.8	48.2
Second test	44.8	56.8	46.1
Difference (Second test-First test)	−1.1	−2.0	−2.1
Lower extremity circumference (cm)			
Paralyzed side, first test	34.2	38.7	40.9
second test	33.9	37.6	40.6
Difference (Second test-First test)	−0.3	−1.1	−0.3
Unaffected side, first test	34.4	38.3	39.3
second test	33.7	36.0	37.6
Difference (Second test-First test)	−0.7	−2.3	−1.7

the three patients. Patients were aged 80, 77 and 73 years. Two patients had cerebral bleeding and one patient had cerebral infarction. There were two men and one woman. The degree of paralysis as assessed by Brunnstrom's system was stage I or II (lower extremity), and in Case B, only slight active movements were possible for both upper and lower extremities (Brunnstrom stage II). All three patients had aphasia, but Case B had severe aphasia and was only able to communicate through eye contact. All three patients had swallowing disorders, and after some period of fasting, transnasal feeding was initiated at 2-8 days after onset. With regard to complications, all patients had hypertension, and Case B had diabetes. Hypertension and diabetes were treated using hypotensive and antidiabetic agents while closely monitoring blood pressure and blood glucose. In addition, all three patients had diarrhea, and Case B had ≥38.0°C fever. While total protein was favorable immediately after admission, it dropped below 6.5 mg/dl at 3-4 days after onset. As to physical activity for the first ten days after onset, all patients stayed in bed. On average, body weight decreased by 1.7 kg and lower extremity circumference by 0.7-1.3 cm.

The following common items were extracted : (1) right hemiplegia ; (2) swallowing disorder ; (3) aphasia ; (4) diarrhea ; (5) precise continuous hypotensive agent injection as main therapy ; (6) total protein on admission was ≥7.0g/dl ; (7) oral intake was initiated ≥ 3 days after admission ; (8) loss of body weight ; (9) loss of lower extremity muscle mass ; and (10) decreased lower extremity circumference. The three patients differed in the following regards : Case A was restless, and it was necessary to restrain the unaffected side (left) ; in Case B, because a family member passively raised the upper extremities forward about two hours a day, upper extremity muscle mass increased ; and Case C raised the unaffected knee on her own.

Discussion

In order to identify the factors related to loss of lower extremity muscle mass, we examined elderly stroke patients with severe right hemiplegia (Brunnstrom stage I or II) in whom our rehabilitation program could not be performed at 1-2 weeks after onset.

Among the three patients, 19 attributes and predictors for low lower extremity muscle mass were analyzed, and ten common factors were extracted. Of these, particularly relevant factors included : severe hemiplegia (Brunnstrom stage I or II) resulting in no movement or minimal active movement on the paralyzed side ; diarrhea ; initially impossible oral intake, and tubal feeding started 3-8 days after onset ; and prolonged accurate microinjection of a hypotensive agent (5-9 days). With regard to movement in Cases A, B and C while lying down, Case A exhibited no intentional or spontaneous movement, but Case C frequently raised the unaffected knee on her own. Patient movements and the degree of decrease in upper and lower extremity muscle mass were analyzed over a 1-week period, and the degree of decrease was low for the areas of the body that were often moved. As has been suggested, loss of lower extremity muscle mass can be minimized by intentionally moving muscles or placing patients in anti-ravity postures[4-5]. However, the factors contributing to patients remaining immobile varied, and as a result, it is necessary to provide care while resolving each issue so that patients can be placed in anti-gravity postures. Based on the results obtained in the present patients, loss of lower extremity muscle mass can be prevented by placing patients in anti-gravity postures, such as sitting or standing, as much as possible, and minimizing the duration of fasting to prevent malnutrition. It is necessary to provide nursing care to resolve these issues.

A limitation in the present study was that only three patients were enrolled. In the future, we plan to continue to investigate factors that increase lower extremity muscle mass and establish nursing techniques to improve the QOL of acute stroke patients.

Conclusions

In three elderly stroke patients with right hemiplegia in whom our rehabilitation program could not be

performed at 1-2 weeks after onset. the factors contributing to the loss of lower extremity muscle mass were investigated. The results identified the following common factors : severe motor dysfunction and hemiplegia resulted in minimal mobility, diarrhea. and delayed initiation of tubal feeding at 3-8 days after onset.

References

1) Odajima N, Ishiai S. Okiyama R, et al : Recovery of atrophic leg muscles in the hemiplegics due to cerebrovascular accidents-Computed tomographic study. Brain Attack 10(1) : 74-78, 1988 (in Japanese with English abstract)

2) Kondo K, Ota T : Changes with time in cross-sectional areas of leg muscles in early stroke rehabilitation patients : disuse muscle atrophy and its recovery. Jpn J Rehabil Med 34(2) : 129-133, 1997 (in Japanese with English abstract)

3) Tamura A, Minagawa T. Takata S, et al ; Effects of intervention with back-lying exercises with bent knees pointing upwards to prevent disuse muscle atrophy in patients with post-stroke hemiplegia. J Nursing Investgation 5(2) : 53-58, 2007

4) Ueda S : Disuse, overuse and misuse and physical therapy in stroke patients : Basic research and clinical studies on disuse, overuse and misuse sign and symptoms. PT journal 27(2) : 76-86, 1993 (in Japanese)

5) Miyoshi S : Early stage rehabilitation of cerebrovascular accident/hemiplegia-a principle and methods, Jap Medical Journal 3549 : 45-49, 1993 (in Japanese)

BRIEF REPORT

Effects of intervention with back-lying exercises with bent knees pointing upwards to prevent disuse muscle atrophy in patients with post-stroke hemiplegia

Ayako Tamura[1], *Takako Minagawa*[1], *Shinjiro Takata*[2], *Takako Ichihara*[1], *Yumi Kuwamura*[1], *Takae Bando*[1], *Hiroko Kondo*[1], *Natuo Yasui*[3], *and Shinji Nagahiro*[3]

[1]*Major in Nursing, School of Health Science, The University of Tokushima, Japan*

[2]*Department of Orthopedies, and* [3]*Department of Neurosurgery, Institute of Health Biosisciences, The University of Tokushima Graduate School, Tokushima, Japan*

Abstract The present study measured lower extremity muscle mass using DXA (Dual energy X-ray Absorptiometry) in order to verify the effectiveness of intervention with a series of movements, including lying hip raise exercise with bent knees pointing upwards, among bedridden patients with post-stroke hemiplegia in the acute post-stroke period. Subjects in the intervention group were required to perform 10 repetitions of a series of back-lying exercises once a day with researchers, in addition to the exercises performed by those in a control group. The first measurement of muscle mass was conducted at three to five days after onset, and the second measurement was conducted seven days after the first. Muscle mass in the lower extremities was reduced by approximately 600 g (decrease rate : 9%) on the paralyzed side and by 280g on the non-paralyzed side (decrease rate : 5%) in one week in the Brunnstrom stage ≤ II subgroup (site of measurement: lower extremities) (n=8) of the control group (n=23). The decrease in muscle mass in the Brunnstrom stage ≤ II subgroup (n=4) of the intervention group (n=15) was approximately 260g on the paralyzed side (decrease rate : 5%) and approximately 280 g (decrease rate : 5%) on the non-paralyzed side. Thus, muscle mass decreased on both sides, and this occurred regardless of degree of paralysis. Comparison of the Brunnstrom stage ≥ III subgroups between the control and intervention groups also confirmed that the decease in muscle mass was smaller in the latter group. Thus, it was confirmed that back-lying exercises combining lower extremity movements, including hip raises with bent knees pointing upwards, prevented the decrease in lower extremity muscle mass on the paralyzed side in post-stroke patients. The present study also suggests that these exercise movements can be applied to preventive care for bedridden patients with other severe diseases.

Key words : effects of intervention, acute post-stroke period, lower extremity muscle, prevent disuse muscle atrophy, hip raise exercises

Introduction

The issue of disuse syndromes, particularly disuse muscle atrophy (muscle mass decrease), in bedridden patients with severe diseases has long been discussed[1,2].

2006年11月10日受付
2007年 1 月17日受理
別刷請求先：田村綾子, 〒770-8509 徳島市蔵本町 3 -18-15
徳島大学医学部保健学科看護学専攻

It has been suggested that developing a rehabilitation program that focuses on the issue of muscle atrophy in the lower extremities during the bedridden period is necessary in order to facilitate early recovery from the bedridden status[3]. Nurses generally provide care, such as daily postural change and maximizing range of motion upon changing clothes, to such bedridden patients.

Although there are many reports on lower extremity muscle atrophy in patients with cerebrovascular disorders[4-9], most of these have evaluated cross sections of the lower extremities using CT and ultrasound echo, and only one study evaluated the whole muscle mass in the lower extremities[10]. Although the need for patient rehabilitation in the acute period has been recognized[3], no standardized practical methods have been established[11].

By establishing a method to prevent the decrease in lower extremity muscle mass, it is possible to not only contribute to QOL improvement in post-stroke patients, but also to help solve the problem of disuse muscle atrophy in bedridden patients with severe diseases. Conducting such a study in the field of nursing should therefore be highly meaningful.

In the present study, we instructed and assisted patients with acute post-stroke hemiplegia in a series of back-lying exercises that combined lower extremity movements, such as hip raises with bent knees pointing upwards. The effectiveness of the intervention was confirmed by examining changes in lower extremity muscle mass.

Objective

In order to verify the effectiveness of a series of exercises we had developed, such as back-lying hip raises with bent knees pointing upwards, to preventing disuse muscle atrophy in patients with post-stroke hemiplegia, we compared changes in lower extremity muscle mass measured using DXA (Dual energy X-ray Absorptiometry) between an intervention group, in which the exercises were introduced during the acute bedridden period, and a control group not performing such exercises.

Definitions of terms

In the present study, the following terms are defined as below : In the acute post-stroke period : This refers to the period "within two weeks from the onset of stroke."

Lower extremity muscle mass : This refers to "the muscle mass in the lower extremities, as measured using DXA." The weight of all muscles from the inguinal region to the toe was measured.

Method

1. Subjects

Subjects consisted of 38 patients who were urgently admitted to hospital "A" due to stroke between May 2005 and July 2006. In these patients without impaired consciousness, hemiplegia was observed, and it was possible to carry out the first measurement at three to four days after onset and the second measurement at 10 to 11 days after onset.

2. Method and Analysis

Disease progress in subjects was managed using the clinical path for strokes at hospital "A." In order to avoid confusion between the control and intervention groups, the study was first conducted in the control group, and subsequently in the intervention group.

Three types of back-lying exercise, including torso twists with bent knees pointing upwards, hip raises with bent knees pointing upwards, and upward kicks with bent knees pointing upwards, in addition to the exercises performed in the control group were each performed ; ten repetitions were performed once a day (at around 4 pm) for approximately 10 minutes in the intervention group. These exercises were introduced with the expectation that contraction of the flexor and extensor muscles in the lower extremities would be facilitated by such movement. The actual movements in these exercises include isotonic muscle contraction (kick up) and closed kinetic chain (hip raise)[12]. The time of exercise was set as above in order to avoid conflict with examinations and treatments, and taking meals. The hip raise exercise with bent knees pointing upwards is

shown in Figure 1. An exercise assistant fixes the knee joints in order to prevent the bottom of the leg on the paralyzed side from sliding, and maintains the angle of knee joint flexion at around 90 to 100°. An angle of knee joint flexion ranging from 90 to 100° allows patients to remain in the easiest posture and prevents the

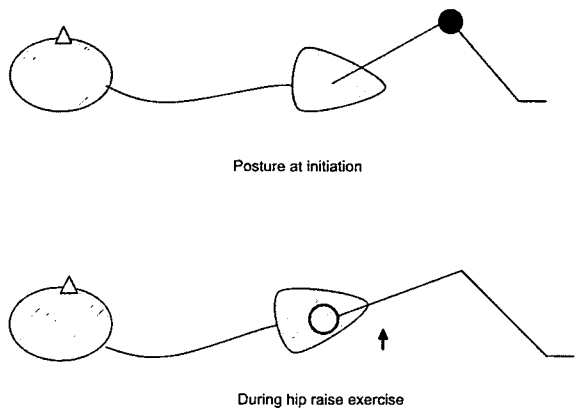

Figure 1. Scheme of hip raise exercise with bent knees pointing upwards in patients with post−stroke hemiplegia
1) The angle of knee joint flexion (●) at initiation should be kept around 90 to 100°.
2) The height of hip elevation should be 5 cm between the trochanteric region on the non- paralyzed side (○) and the bed surface.

paralyzed leg from sliding. The hip was raised until the trochanteric region on the non-paralyzed side was elevated to 5 cm. In order to ensure consistency and accuracy, we practiced this exercise program in healthy individuals prior to using it with patients. One researcher was exclusively involved in providing intervention exercises in order to eliminate differences in the contents and methods of the study. Furthermore, persons in charge of examinations, including DXA and Brunnstrom, and exercise supporters concealed individual test results and status of patients in order to maintain a clear border between researchers and evaluators. Thus, bias in the research results was minimized.

DXA (QDR Delphi (Hologic Inc. USA)) was used for measurement of muscle mass in the left and right lower extremities. Muscle mass evaluation was performed by one researcher who was skilled in DXA. The first measurement was conducted at 3 to 5 days after onset, and the second measurement was conducted on the 7 th day after the first measurement. Brunnstrom stage was determined by one researcher for all subjects on the day of the first DXA measurement.

For data analysis, subjects were divided into Brunnstrom stage I to II subgroup with mostly immobile patients (Brunnstrom stage ≤ II group) and a Brunnstrom stage III to V subgroup with relatively mobile subjects (Brunnstrom stage ≥ III group), based on degree of motion of the paralyzed lower extremity. Regarding the difference between the first and second measurements of muscle mass, average values and decrease rates were calculated for the paralyzed and non-paralyzed side in each group. Decrease rates were calculated based on the following formula: (muscle mass on first measurement−muscle mass on second measurement)/ muscle mass on first measurement×100. A Wilcoxon matched-pair signed-rank test was performed to analyze the data using SPSS 11.5 for Windows, with statistical significance being set at $P<0.05$.

3. Ethical considerations

The present study was conducted after receiving the approval of the Ethics Committee for Clinical Research at Tokushima University Hospital. The contents of the study were explained to the subjects and their families. Upon verbal and written explanation that participation was voluntary, that nobody would be disadvantaged in medical treatment and nursing due to discontinuation or lack of participation in the study, and that privacy would be protected, agreement to participate was obtained in writing.

Results

Table 1 shows the clinicodemographic background data of the 38 subjects (23 in the control group and 15 in the intervention group). In the control group, the average age of the subjects was 65.1 years (SD 13.2). Cause of stroke (primary disease) was cerebral infarction in 12 subjects, and intracranial hemorrhage in 11. Hemiplegia was left-sided in 14 subjects, and right-sided in nine. The Brunnstrom stage ≤ II subgroup comprised eight subjects, while the Brunnstrom stage ≤ III subgroup comprised 15 subjects. In the intervention group, the average age of the subjects was 67.0 years (SD12.0).

Cause of stroke was cerebral infarction in 12 patients and intracranial hemorrhage in three. Hemiplegia was left-sided in 11 subjects, and right-sided in four. The Brunnstrom stage ≤ II subgroup comprised four subjects, while the Brunnstrom stage ≥ III subgroup comprised 11 subjects.

Changes in muscle mass and decrease rates for each Brunnstrom subgroup in the intervention and control groups are shown in Table 2. The decrease in muscle mass in the control group was 292g (SD 239) on the paralyzed side and 123 g (SD 277) in the Brunnstrom stage ≥ III subgroup. The decrease rate was 5.0% (SD 4.2) on the paralyzed side and 2.0% (SD4.5) on the non-paralyzed side; thus, a significant difference was observed ($P<0.05$). In the Brunnstrom stage ≤ II subgroup, the decrease was 609g (SD 233) and 316 g (SD 303) on the paralyzed and non-paralyzed sides, respectively. Although the difference in decrease rate between the paralyzed and non-paralyzed sides, 9.0% (SD 3.5) and 5.0% (SD4.2), respectively, was not significant, a clear trend ($P=0.07$) was observed.

On the other hand, the decrease in muscle mass was only 77 g (SD 295) on the paralyzed side and 131 g (SD 334) on the non-paralyzed side in the Brunnstrom stage ≥ III subgroup of the intervention group. The decrease rate was almost identical between the paralyzed and non-paralyzed sides (1.6% (SD4.4) and 1.9% (SD 5.0), respectively), and no significant difference was observed. The same trend was observed in the Brunnstrom stage ≤ II subgroup; no significant difference was observed between the paralyzed and non-paralyzed sides in decrease in muscle mass, 267g (SD 203) and 282 g (SD 406), or in decrease rate, 4.8% (SD 4.0) and 4.8% (SD6.5), respectively.

Table 1 Background of subjects

		control group N=23	intervention group N=15	Total N=38
Gender	Male	14	10	24
	Female	9	5	14
Age	40–49	3	1	4
	50–59	7	3	10
	60–69	2	3	5
	70–79	7	6	13
	Above 80	4	2	6
	Average (SD)	65.1(SD13.2)	67.0 (SD12.0)	
Primary disease	Cerebral infarction	12	12	24
	Intracranial hemorrhage	11	3	14
Side of paralysis	Right	9	4	13
	Left	14	11	25
Degree of hemiplegia Brunnstrom stage (lower extremities)				
	stage III ~ V	15	11	26
	stage I ~ II	8	4	12

Table 2 Average decrease and rate of decrease of lower extremity muscle mass

	Brunnstrom stage		Muscle amount decrease (g) Mean (SD)	Decrease rate (%) Mean (SD)	Wilcoxon matched-pair signed-rank test
control group	stage III or above				
	Paralyzed side	15	292(239)	5.0(4.2)	*
	Non-paralyzed side	15	123(277)	2.0(4.5)	
	stage II or below				
	Paralyzed side	8	609(233)	9.0(3.5)	n.s.
	Non-paralyzed side	8	316(303)	4.8(3.9)	
intervention group	stage III or above				
	Paralyzed side	11	77(295)	1.6(4.4)	n.s.
	Non-paralyzed side	11	131(334)	1.9(5.0)	
	stage II or below				
	Paralyzed side	4	267(203)	4.8(4.0)	n.s.
	Non-paralyzed side	4	282(406)	4.8(6.5)	

(* : $P<0.05$ n.s.: not significant)

Discussion

With the recent progress in our understanding of rehabilitation programs for post-stroke patients in the acute period, the importance of

providing such rehabilitation in the acute period is being recognized. However, post-stroke bedridden patients in the acute period after onset to are treated according to 2 contradicting methods, rest based on the acute period management and exercise for prevention of disuse syndromes, and thus tend to be maintained in the bedridden status. Furthermore, the need for rehabilitation has been recognized[10]according to the actual situation of disuse muscle atrophy in the lower extremity muscles in post-stroke patients[3,10]. However, no standardized practical methods have been established. By actively providing a rehabilitation program for bedridden patients in the acute stage, it is possible to break the vicious circle of disuse syndromes[13], and contribute to improvement of QOL of patients by helping to reduce the hospitalization period and return to work.

Studies using several conventional evaluation methods for disuse muscle atrophy in post-stroke patients have been reported, including methods to estimate the decrease in lower extremity muscle mass based on muscle cross sections using CT[3,9]and ultrasound echo[6,7], and a method to measure muscle mass only on the healthy side using a dynamometer[4]. The DXA method utilized in the present study was originally used to measure bone density. Because the method allows measurement of individual body components (bone, muscle and fat) in the right and left lower extremities[14], and the measurement error is as low as 0.2-2.2%[14,15], it is possible to accurately evaluate the entire muscle mass of left and right lower extremities.

For intervention, exercises were designed to facilitate muscle contraction of flexor and extensor muscles in the lower extremities, and to consist of movements in the daily lives of bedridden patients. By including the series of movements described above, which are associated with movements with bent-knees pointing upwards and kick-up movements, it was expected that isotonic muscle contraction (kick up) and closed kinetic chain (hip raise)[12] would be activated. Although a short period of time, 10 minutes a day, was spent on exercise, the exercise load was not insufficient according to the report by Hettinger, et al.[16], which suggested that performing muscle contraction exercises for 6 seconds a day increased muscle mass, and was equivalent to the level of training reported by Ichihashi, et al.[17].

In the intervention group, changes in muscle mass on the paralyzed and non-paralyzed sides were almost equivalent in both the Brunnstrom stage ≥ Ⅲ subgroup and the Brunnstrom stage ≤ Ⅱ subgroup, and the decrease rate was as low as 2 % and 5 % in the Brunnstrom stage ≥ Ⅲ subgroup and Brunnstrom stage ≤ Ⅱ subgroup, respectively. On the other hand, differences in muscle mass decrease were observed between the paralyzed side, approximately 609 g (9.0%), and non-paralyzed side, approximately 300 g (5.0%), in the Brunnstrom stage ≤ Ⅱ subgroup of the control group. Standard deviations were large in the present study. This was assumed to be because muscle changes were expressed as a difference between the muscle mass in the first measurement and second measurement, and there were some cases in which muscle mass increased. Such cases were particularly observed in the Brunnstrom stage ≥ Ⅲ subgroup of the intervention group, and increase rates were highly diverse. Such issues should be examined in future research.

Having post-stroke patients with hemiplegia perform a series of back-lying exercises, including hip raises with bent knees pointing upwards, in intervention makes them realize that such movements are "preferable movements ; "therefore, it was assumed that the activity level of the lower extremities with bent knees pointing upwards was increased in bedridden patients. Because objective observation of activity levels of the lower extremities with bent knees pointing upwards was not achieved in the present study, further study investigating this issue is needed. The present study revealed that intervention reduced the loss of muscle mass in the lower extremities ; however, it remains unclear which parts exhibited less reduction in muscle mass. This should also be investigated in future research.

It is expected that the series of exercises used in the present study is applicable to not only post-stoke patients but also to bedridden patients with severe diseases. By conducting further studies in which frequency and intensity of the series of back-lying movements are observed in daily life, better movement sup-

port methods could be designed.

References

1) Masakado Y, Chino N : The current states of stroke rehabilitation in Japan. Brain and Circuration 5 (4) : 317-322, 2000 (in Japanese)

2) Miyoshi S : Early rehabilitation of cerebral apoplexy. Japanese medical business new account 3596 : 45-49, 1993 (in Japanese)

3) Kotake T, Dohi N : Study of trunk and extremity muscles in the hemiplegics due to cerebrovascular accidents using CT findings. Jpn J Lihabil Med 28 (8) : 607-612, 1991 (in Japanese with English abstract)

4) Okawa Y, Ueda S : The disuse muscle atrophy in the hemiplegic patients : the muscle weakness in the "unaffected" extremities. Jpn J Lihabil Med 25(3) : 143-147, 1988 (in Japanese with English abstract)

5) Odajima N, Ishiai S, Okiyama R, et al : Recovery of atrophic leg muscles in the hemiplegics due to cerebrovascular accidents-Computed tomographic study. Brain Attack 10(1) : 74-78, 1988 (in Japanese with English abstract)

6) Umahara T, Sasaki A, Imamura T, et al : Ultrasound Imaging of anterior tibial muscle in hemiplegics due to cerebrovascular disease. Jpn J Med Imaging 10 (1) : 38-43, 1991 (in Japanese with English abstract)

7) Umahara T, Kitaoka T, Imamura T, et al : Ultrasound Imaging of anterior tibial muscle in hemiplegics due to cerebrovascular disease. Jpn J Lihabil Med 29(2) : 145-151, 1993 (in Japanese with English abstract)

8) Kondo K : Muscle fiber conduction velocity study as an indicator of muscle atrophy in the hemiplegia. Jpn J Lihabil Med 36(7) : 477-484, 1999 (in Japanese with English abstract)

9) Kondo K, Ota T : Changes with time in cross-sectional areas of leg muscles in early stroke rehabilitation patients : disuse muscle atrophy and its recovery. Jpn J Rehabil Med 34(2) : 129-133, 1997 (in Japanese with English abstract)

10) Tamura A, Ichihara T, Takata S, et al : Changes in lower extremity muscle mass among bedridden patients with post-stroke hemiplegia in the acute post-stroke period. J Nursing Investigation 5(1) : 18-21, 2006

11) Shinohara Y, Yoshimoto T, Fukuuchi Y, et al eds. : The joint committee on guidelines for the management of stroke : The Japanese guidelines for the management of stroke 2004, Kyowakikaku Inc, Tokyo, 2004, pp. 170-180

12) Hinohara S, Imura H, eds. : Symphonia medica nursing 27, Rihabilitationand exercise therapy, Nakayama Bookstore, Tokyo, 2002, pp. 245

13) Ueda S, Disuse : overuse and misuse and physical therapy in stroke patients ; basic research and clinical studies on disuse, overuse and misuse sign and symptoms. PT journal 27(2) : 76-86, 1993 (in Japanese)

14) Lohman T : Dual energy X-ray Absorptiometry. In : Rose A, Heymsfield S, Lohman T, eds, Human Body Composition, Human Kinetics Publishers Inc., Japanese translation, Komiya S. Taishukan, 2001, pp.119-138

15) Kawakatu M, Shimogaki K, Korenari Y, et al : Body compositional analysis by Dual energy X-ray Absorptiometry : basic evaluation. A M J 23(2) : 65-66, 1991 (in Japanese)

16) Hettinger T, Muller E A : Muskeleistung und muskeltraining. Internationale Zeitschrift fur Angewandte Phisiologie Einschleisslich Arbeitsphysiologie 15 : 111-126, 1953

17) Ichihashi N, Itoh H, Sakamoto T, et al : Influence of non-exercise and exercise on knee extensor and flexor during. Physicotherapeutics 18(4) : 397-403, 1991 (in Japanese with English abstract)

Clin Rheumatol (2007) 26:1284–1292
DOI 10.1007/s10067-006-0495-8

ORIGINAL ARTICLE

Distinct expression of mast cell tryptase and protease activated receptor-2 in synovia of rheumatoid arthritis and osteoarthritis

Shunji Nakano · Takuya Mishiro · Shigeyuki Takahara · Hiromichi Yokoi · Daisuke Hamada · Kiminori Yukata · Yoichiro Takata · Tomohiro Goto · Hiroshi Egawa · Susumu Yasuoka · Harumi Furouchi · Katsuya Hirasaka · Takeshi Nikawa · Natsuo Yasui

Received: 21 October 2006 / Revised: 13 November 2006 / Accepted: 13 November 2006 / Published online: 5 January 2007

Abstract The objective of this study is to examine the differential expression of mast cell tryptase and its receptor, protease-activated receptor-2 (PAR-2), in the synovium and synovial fluid of patients with rheumatoid arthritis (RA) and osteoarthritis (OA). Biochemical and immunohistochemical analyses were performed to determine whether the trypsin-like protease in the synovium is identical to mast cell tryptase. The effects of mast cell tryptase on the proliferation of synovial fibroblast-like cells (SFCs) and the release of IL-8 thereof were evaluated by the [^{3}H]-thymidine incorporation and ELISA, respectively. The trypsin-like protease in the synovium of RA patients was identical to human mast cell tryptase, which was composed of two subunits: 33 and 34 kDa. The 33- and 34-kDa proteins are different glycosylated forms of the 31-kDa protein, which was unglycosylated. Mast cell tryptase activity in RA synovial fluid was significantly higher than that in OA synovial fluid, while their activities and expression in the synovium were similar. Expression of PAR-2 mRNA in the synovium was higher in RA than in OA. Mast cell tryptase containing the unglycosylated 31-kDa subunit was the predominant form in synovial fluid. RA patients had higher amounts of this subunit in their synovial fluid than OA patients. Mast cell tryptase and PAR-2 activating peptide stimulated the proliferation of SFCs and release of IL-8 from these cells. Mast cell tryptase secretion into RA synovial fluid is higher than OA synovial fluid. Mast cell tryptase in synovial fluid stimulates the proliferation of SFCs and the release of pro-inflammatory cytokines via PAR-2, which may contribute to exacerbation of synovitis in RA.

Keywords Mast cell tryptase · Osteoarthritis · Protease-activated receptor-2 · Rheumatoid arthritis · Synovial fluid · Synovium

S. Nakano · T. Mishiro · S. Takahara · H. Yokoi · D. Hamada · K. Yukata · Y. Takata · T. Goto · H. Egawa · N. Yasui (✉)
Department of Orthopedics, Institute of Health Biosciences,
The University of Tokushima Graduate School,
3-18-15 Kuramoto,
Tokushima 770-8503, Japan
e-mail: nyasui@clin.med.tokushima-u.ac.jp

S. Yasuoka
Department of Internal Medicine, Hakuai Kinen Hospital,
Tokushima 770-8023, Japan

H. Furouchi · K. Hirasaka · T. Nikawa
Department of Nutrition, Institute of Health Biosciences,
The University of Tokushima Graduate School,
Tokushima 770-8503, Japan

Introduction

Rheumatoid arthritis (RA) is usually associated with synovitis characterized by the presence of synovial fluid that frequently contains various chemical mediators, degradation products from articular structures, and exudates from the blood stream. Therefore, synovial fluid has been suggested to reflect, to some extent local, inflammatory reactions in the joints affected by RA, and hyperplasia of the synovium producing synovial fluid is one of the pathological bases of RA.

A number of investigations [1–9] have shown that mast cells infiltrate into the synovium in patients with arthritic diseases such as RA and osteoarthritis (OA) and that products released from mast cells perpetuate the inflammatory process and ultimately destroy the joint. Mast cell tryptase, a trypsin-like serine protease [10, 11], is a major component of such products and stimulates inflammation and proliferation of various types of cells via protease activated receptor-2 (PAR-2), a receptor for the trypsin family of proteases such as trypsin and mast cell tryptase [12–16]. However, there have been few studies demonstrating the differences of mast cell tryptase and PAR-2 expression in the inflamed synovium of inflammatory joint diseases.

In this study, we first confirmed whether trypsin-like protease partially purified from the synovium of RA patients was identical to human mast cell tryptase. Then, we examined the expression of mast cell tryptase and PAR-2 in RA and OA to elucidate the pathological roles of mast cell tryptase and PAR-2, which were highly expressed in articular lesions of RA patients. Furthermore, we investigated the effect of mast cell tryptase on the proliferation of synovial fibroblast-like cells (SFCs) and IL-8 release from SFCs in RA patients. Our results suggest that the higher secretion of mast cell tryptase from mast cells recruited into the synovium in RA patients may, at least in part, contribute to a more severe synovitis in RA compared with OA.

Materials and methods

Patients' characteristics

The diagnosis of RA was based on the American Rheumatism Association 1987 revised criteria [17]. The patients with OA who participated in this study met the clinical and radiological diagnostic criteria [18]. All patients with OA had effusions in their knee joints, and the synovial fluids were clear and highly viscous. The age, sex, erythrocyte sedimentation rate (ESR), serum C-reactive protein (CRP) concentration, and rheumatoid factor (RF) status of patients and the medication they were receiving are shown in Table 1.

Synovial fluid and synovium specimens

Synovial fluids were collected from the knee joints of RA and OA patients by puncture at Tokushima University Hospital. They were then centrifuged at 1,000×*g* for 10 min at 4°C within 1 h after being collected. The supernatants were immediately frozen and stored at −80°C until use. Synovial fluids were also collected from four healthy volunteers and used as control. Synovium samples were obtained from RA and OA patients during joint surgery. After washing with phosphate-buffered saline (PBS), the tissues were homogenized in a ninefold volume of 50 mM Tris–HCl buffer (pH 7.5) containing 0.5 M NaCl with a Polytron homogenizer on ice for 1 min. After centrifugation at 1,000×*g* for 10 min at 4°C, the supernatants were collected and used as synovial extracts. Control synovia were similarly prepared from patients with intracapsular hip fracture.

The study protocol was approved by the Ethics Committee of The University of Tokushima Graduate School. All the patients and volunteers who participated in this study gave their written informed consent.

SFC culturing and treatment with reagents

SFCs were cultured and maintained as described previously [19]. Fresh synovia obtained from RA patients were cut to pieces of about 2 mm in diameter and cultured in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum, 100 U/ml penicillin G sodium, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The medium was replaced twice a week until SFCs had grown to about 60% confluence. They were treated with our previously purified human lung mast cell tryptase [20] or a PAR-2 activating peptide (H-Ser-Leu-Ile-Gly-Lys-Val-NH_2; Bachem AG, Bubendorf, Switzerland) at the indicated concentrations. After treatment with the reagents, cell proliferation rate and IL-8 release from SFCs were measured on days 1 and 2, respectively, as described below. Cells from the first three passages were used in this study.

Table 1 Clinical characteristics and drug treatment of patients

Patients	Number (male/female)	Age (year)	ESR (mm/h)	CRP (mg/dl)	RF (IU)
RA[a]	43 (7/36)	63.0 (28.6–81.6)	58.9 (12–134)	3.35 (0.21–10.59)	141 (3–820)
OA[a]	45 (14/31)	70.0 (41.0–86.6)	WNL	WNL	WNL

The values are expressed as mean with the range in parenthesis.
WNL Within normal limit
[a] Almost all of the RA patients were medicated: corticosteroids, 18; disease-modifying antirheumatic drugs, 41; nonsteroidal anti-inflammatory drugs (NSAIDS), 27. Twenty OA patients were being treated with NSAIDs.

Protease activity assay

Trypsin-like activity was measured by spectrofluorophotometry using a synthetic fluorogenic peptide (Peptide Institute, Osaka, Japan), Boc-Gln-Ala-Arg-MCA as the substrate, as described previously [20]. Synovial fluid and synovial tissue extract were diluted tenfold and 100-fold, respectively, with saline and used as the enzyme source. One unit of enzymatic activity was defined as the amount of enzyme that released 1 μmol of 7-amido-4-methylcoumarin per minute. The inhibitory activity of antipain, leupeptin, and soybean trypsin inhibitor (Sigma, St. Louis, MO) for partially purified trypsin-like protease was also measured. The reaction mixtures containing these inhibitors at various concentrations were allowed to stand at room temperature for 10 min and the trypsin-like activity was measured in the same manner as described above.

Partial purification of trypsin-like protease from synovium

Synovial tissue extract (1 mg protein) was applied to a gel filtration column (2.2×65 cm) of Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden), equilibrated and eluted with 50 mM Tris–HCl buffer (pH 7.5) containing 0.5 M NaCl at a flow rate of 0.5 ml/min. Protein concentration was monitored with a UV detector at 280 nm. The fractions containing trypsin-like activity, which were designated as Fraction I (Fig. 1a), were collected and concentrated by centrifuged filtration (cutoff molecular mass≈30,000; Millipore, Billerica, MA). Human mast cell tryptase, previously purified from the lung tissue [20], was also fractionated on the same gel filtration column chromatography.

Western blot analysis

The synovial fluid (80 μg protein/lane) and synovial extracts (40 μg protein/lane) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% gel and transferred to a polyvinylidene difluoride membrane at 35 mA for 6 h at 4°C. The membrane was blocked with 4% skim milk and then incubated with a polyclonal rabbit anti-human tryptase antibody (Biogenesis, Poole, UK) for 1 h at 25°C. The

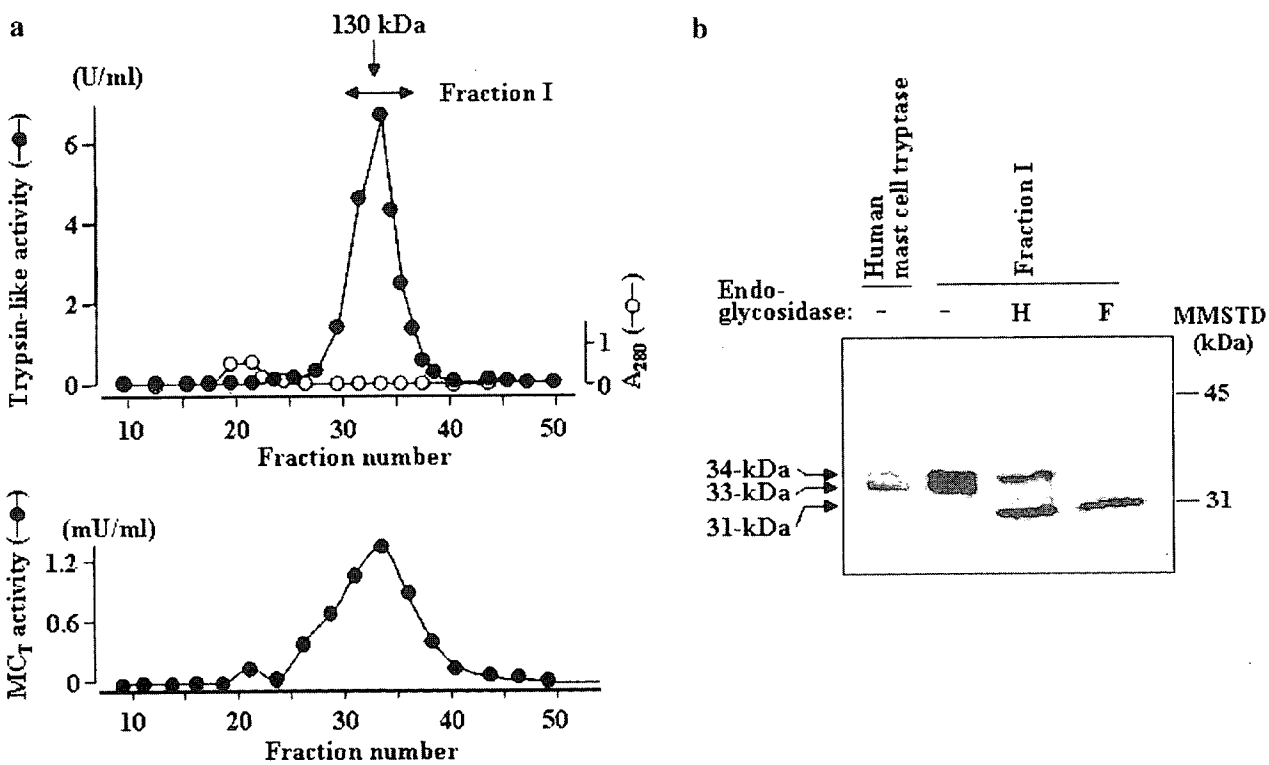

Fig. 1 Identification of synovial trypsin-like protease to mast cell tryptase. (**a**) Homogenates (1 mg protein) from the synovium of an RA patient or purified human mast cell tryptase (3 μg) were subjected to gel filtration chromatography on a column of Sephadex G-200. The tubes containing tryptase-like activity, designated as Fraction I (Fraction No. 30–37), were collected and concentrated. The trypsin-like activity of 0.1 ml of each fraction was measured with 50 μM Boc-Gln-Ala-Arg-MCA as the substrate. (**b**) Concentrated Fraction I and purified mast cell tryptase were subjected to Western blot analysis using an anti-human mast cell tryptase antibody. Respective samples digested with endoglycosidases H and F were also subjected to Western blotting in the same manner. *MMSTD* Molecular mass standards

bound antibodies were detected with a suitable secondary antibody (Amersham Biosciences, Buckinghamshire, UK) using the enhanced chemiluminescence system (Amersham Biosciences). We used albumin as an internal standard protein because an excessive amount of albumin in the synovial fluid interfered with the detection of β-actin by Western blotting (data not shown).

Immunohistochemical analysis

Synovium (approximately 5 mm in diameter) taken at random from several different areas of the joint were subjected to immunohistochemical analysis for mast cell tryptase as described previously [21]. Briefly, samples were frozen in a glycerol-base mounting medium (Miles Laboratories, Naperville, IL), and cut into 8-μm-thick sections with a cryostat. The sections were incubated with 3% hydrogen peroxide for 20 min at 25°C to inactivate endogenous peroxidase. After washing with PBS for 10 min, the sections were treated with avidin D and biotin, followed by further incubation at 4°C overnight with a biotin-conjugated monoclonal anti-human tryptase antibody (Promega, Madison, WI) diluted at 1:5,000 in PBS containing 1% non-immune goat serum. The bound antibodies were detected with a Vectastain ABC kit (Vector Lab, Burlingame, CA) and 3, 3′-diaminobenzidine (Sigma) as the substrate.

Real-time reverse transcription and polymerase chain reaction

Total RNA was isolated from synovium with an acid guanidinium thiocyanate–phenol–chloroform mixture (NipponGene, Osaka, Japan) [21]. Real-time reverse transcription and polymerase chain reaction (RT-PCR) with SYBR green dye was performed by using an ABI 7300 real-time PCR system (Applied Biosystems, Foster City, CA) as described previously [21]. The following oligonucleotide primer sets were used: 5′-CTGCATCGACCCC TTTGTCT-3′ and 5′-GGAGAGCGTTCTTTGCATGAT-3′ for human PAR-2; 5′-ACCCAGAAGACTGTGGATGG-3′ and 5′-TTCAGCTCTGGGATGACCTT-3′ for human glyceraldehyde-3-phosphate dehydrogenate (GAPDH).

Other biochemical analyses

Glycosidase digestion of mast cell tryptase was performed as described previously [22]. Twenty micrograms of partially purified trypsin-like protease (Fraction I) or human lung mast cell tryptase was incubated with 5 IU endoglycosidase H (Wako Pure Chemical Industries, Osaka, Japan) or endoglycosidase F (Roche Diagnostics, Tokyo, Japan) for 3 h at 37°C. The proliferation of SFCs was estimated by the incorporation of [^{3}H]-thymidine as described previously [23]. The concentration of IL-8 in the culture medium was measured with an IL-8-specific enzyme-linked immunosorbent assay (ELISA) kit (Endogen, Woburn, MA) as described previously [23]. The protein concentration was determined by Lowry's method with bovine serum albumin as a standard [24].

Statistical analysis

Data were expressed as the mean±SD or SE. Statistical significance of differences between groups was assessed by nonparametric Mann–Whitney test. A P value of less than 0.05 was considered to indicate a statistically significant difference.

Results

Identification of trypsin-like protease in RA to mast cell tryptase

Gel filtration column chromatography of synovium extracts from RA patients showed that trypsin-like activity was prominently detected in Fraction No. 30–37, as shown in Fig. 1a. Fraction No. 34 contained the peak activity and corresponded to an apparent molecular mass of 134 kDa. The gel filtration profile of human mast cell tryptase activity on the same gel chromatography column showed a similar pattern (bottom panel in Fig. 2a), suggesting that the trypsin-like protease had a similar apparent molecular mass to human mast cell tryptase. Western blotting with an anti-tryptase antibody revealed that both trypsin-like protease from the synovium of RA patients and human mast cell tryptase were immunoreactive with an anti-tryptase antibody and consisted of two major subunits with molecular masses of 33 and 34 kDa (Fig. 1b). When the protein was treated with endoglycosidase H, only the 33-kDa band of the purified trypsin-like proteases shifted to a position corresponding to the 31-kDa band. In contrast, treatment with endoglycosidase F decreased the molecular masses of both 34- and 33-kDa subunits to 31 kDa (Fig. 1b).

The substrate and inhibitor specificities and activities of the trypsin-like protease were also studied. Among the tested substrates, trypsin-like protease showed the highest hydrolytic activity for Boc-Gln-Ala-Arg-MCA as did the purified human mast cell tryptase (Table 2). Hydrolytic activities of trypsin-like protease for Boc-Phe-Ser-Arg-MCA and Boc-Val-Pro-Arg-MCA were also similar to those of human mast cell tryptase. Trypsin-like protease and mast cell tryptase showed little or no activity for other serine protease and collagenase substrates. In contrast, trypsin-like protease from RA patients and purified mast

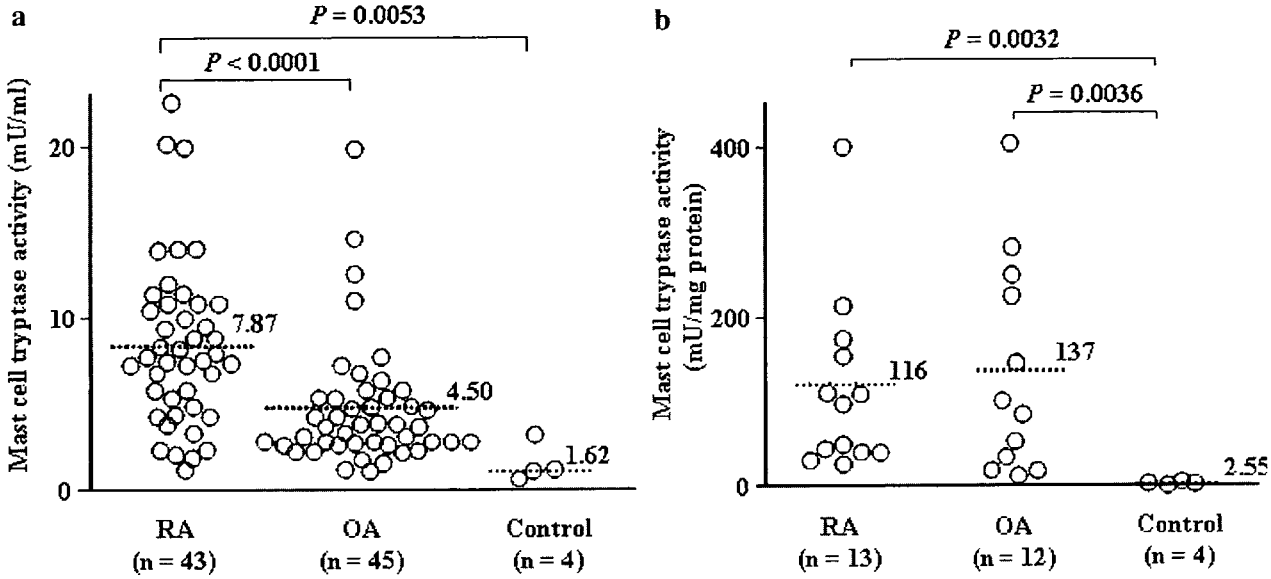

Fig. 2 Tryptase activity in synovial fluids and synovium from RA and OA patients (**a**, **b**). Synovial fluid (**a**) and synovial extract (**b**) were prepared from patients with RA, OA, or intracapsular hip fracture and healthy volunteers (control). Mast cell tryptase activity in each sample was measured by spectrofluorophotometry, using Boc-Gla-Ala-Arg-MCA as the substrate, as described in the "Materials and methods" section. *Horizontal dotted lines* show the mean value in each group. *P* values indicate a statistically significant difference

cell tryptase were completely inhibited by antipain and leupeptin, whereas soybean trypsin inhibitor failed to inhibit both enzymes (Table 2). Based on the results, we concluded that trypsin-like protease in the synovia of RA patients was identical to mast cell tryptase.

Expression of mast cell tryptase and PAR-2 in synovia of RA and OA patients

Mast cell tryptase activity in the synovial fluid was significantly higher in the RA group compared to the OA

Table 2 Substrate and inhibitor specificities and activities of the partial purified trypsin-like protease from RA synovium

Substrate	Inhibitor (concentration)	Relative activity (%)	
		Purified trypsin-like protease	Human mast cell tryptase
Boc-Gln-Ala-Arg-MCA		100	100
Boc-Phe-Ser-Arg-MCA		41.4	29.8
Boc-Val-Pro-Arg-MCA		25.1	19.2
Boc-Ile-Glu-Gly-Arg-MCA		0.32	0
Boc-Val-Leu-Lys-MCA		4.95	0.03
Glt-Gly-Arg-MCA		0.33	0.01
Suc-Ala-Ala-Pro-Phe-MCA		0	0
Suc-Gly-Pro-Leu-Gly-Pro-MCA		0	0
Suc-Ala -Pro-Ala-MCA		0	0
Boc-Gln-Ala-Arg-MCA			
Antipain	(1×10^{-6})	59.0	75.9
	(1×10^{-5})	10.0	20.4
	(1×10^{-4})	1.2	2.7
Leupeptin	(1×10^{-6})	50.7	60.0
	(1×10^{-5})	8.8	12.5
	(1×10^{-4})	1.0	1.5
Soybean trypsin inhibitor	(1×10^{-6})	100	100
	(1×10^{-5})	100	100
	(1×10^{-4})	100	100

The relative activity is a percentage of the enzymatic activity compared with the activity obtained for Boc-Gln-Ala-Arg-MCA (100%).

group and the control group (Fig. 2a). Mast cell tryptase activities in synovium extracts from RA and OA patients were similar, although they were significantly higher than that in the control group (Fig. 2b).

Immunohistochemical analysis showed that the number of cells that stained positive for mast cell tryptase were higher in the synovium of RA and OA patients compared with control synovium (Fig. 3a); this is consistent with mast cell tryptase activity in synovial extracts. Furthermore, Western blotting revealed that in synovial fluid, a 31-kDa band that was an unglycosylated form of the 34- and 33-kDa subunits of mast cell tryptase (Fig. 1b) was the predominant form, while the synovium expressed mainly the 34- and 33-kDa subunits of mast cell tryptase (Fig. 3b). Interestingly, the 31-kDa subunit of mast cell tryptase was more highly concentrated in the synovial fluid from RA patients compared with that from OA patients. The 31-kDa subunit of mast cell tryptase was found in the synovium from RA patients, although it was not detected in the synovium from OA patients. We also measured mRNA transcripts of PAR-2, a mast cell tryptase receptor, in the synovium of RA and OA patients. The amount of PAR-2 mRNA transcript in the synovium of RA patients was statistically significantly higher than that from OA patients (Fig. 3c).

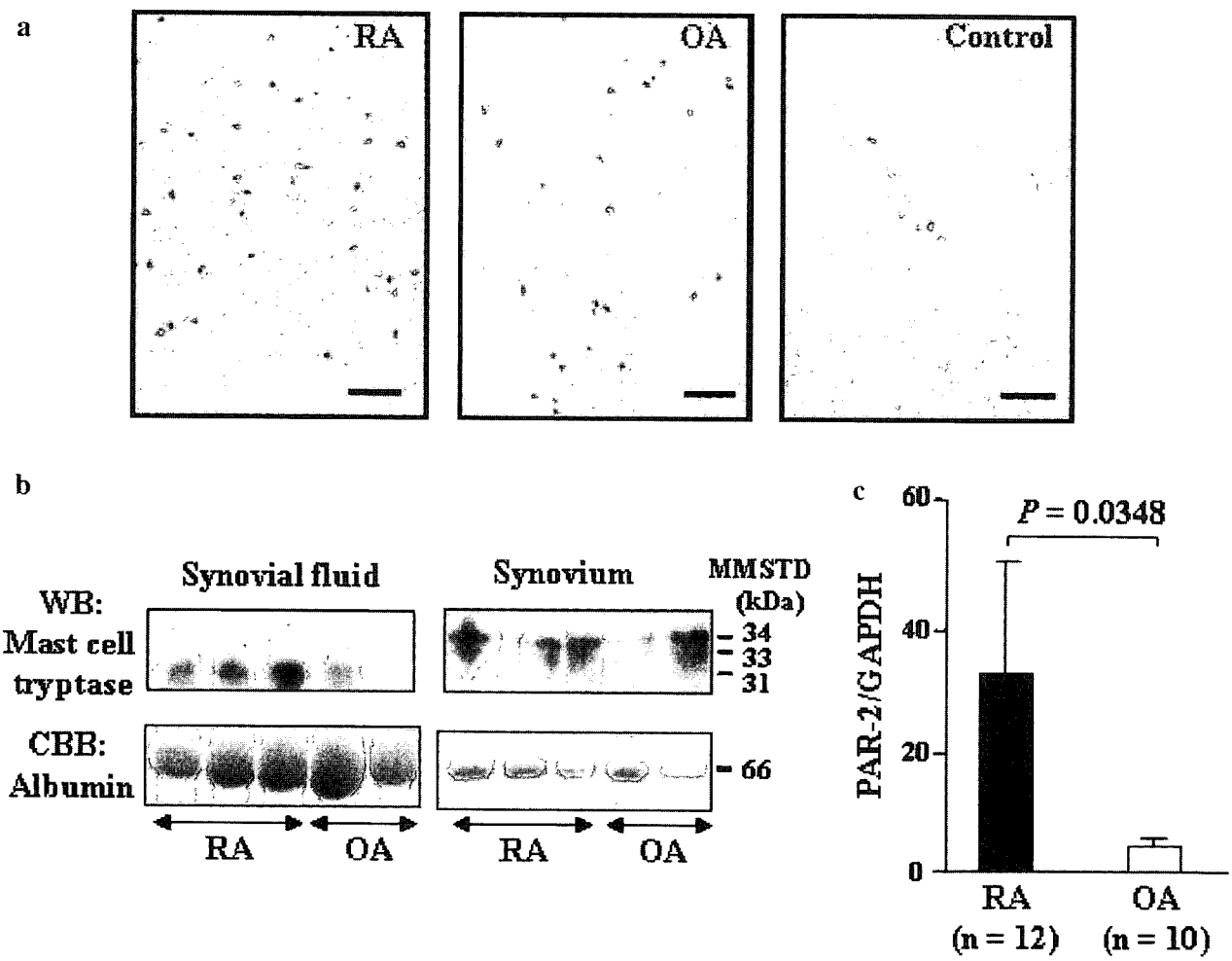

Fig. 3 Amounts of mast cell tryptase protein and of PAR-2 transcripts in synovial tissues. **a** Sections of synovium from patients with RA, OA, or intracapsular hip fracture were immunohistochemically stained with an anti-mast cell tryptase antibody. Immunopositive cells indicate mast cell accumulated into synovial tissues. Similar results were obtained in three independent experiments. The *horizontal bar* in each panel represents 150 μm. **b** Samples were prepared from synovial fluid and synovium from RA and OA patients. Extracts (80 μg protein/lane from synovial fluid and 40 μg protein/lane from synovium) were subjected to SDS-PAGE followed with Western blotting (*WB*) for mast cell tryptase or Coomassie brilliant blue (*CBB*) staining. The apparent molecular weight of albumin was 66 kDa. *MMSTD* Molecular mass standards. **c** Total RNA prepared from synovium of RA and OA patients was subjected to real-time RT-PCR. The concentration of PAR-2 transcript was shown as the ratio of amounts of PAR-2 transcript to that of human GAPDH. Data are the mean (*bar* denotes SE). *P* value of 0.0348 indicates a statistically significant difference

Effects of mast cell tryptase and PAR-2 activating peptide on the proliferation and proinflammatory cytokine release from cultured SFCs

Lastly, we examined effects of mast cell tryptase on the proliferation of SFCs and IL-8 release from SFCs of RA patients to determine whether increased mast cell tryptase in synovial fluid was involved in the exacerbation of synovitis in RA. Human mast cell tryptase significantly stimulated proliferation of SFCs and release of IL-8 from these cells in a dose-dependent manner (Fig. 4a). The expression of PAR-2 transcripts in the synovium from RA patients was statistically significantly higher than that from OA patients (Fig. 3c), indicating that these stimulatory effects of mast cell tryptase were mediated by PAR-2-dependent pathway in SFCs. To confirm this hypothesis, SFCs were also treated with PAR-2 activating peptide, a PAR-2-specific activator. The PAR-2 activating peptide stimulated IL-8 release of SFCs from RA patients with comparable effectiveness to human mast cell tryptase, but not from OA patients (Fig. 4b).

Discussion

Heterogeneity of tryptase has been observed at both the structural and functional levels of the protein [10, 11, 25–32]. Studies of this enzyme have indicated microheterogeneity of the subunits, with molecular masses ranging from 31 to 38 kDa on SDS/polyacrylamide gels sometimes as a broad, diffuse band and sometimes as discrete bands. The present study also showed that synovial mast cell tryptase in inflammatory joint diseases was an oligomer of 31–34 kDa subunits. Consistent with the results of mast cell tryptase activity, mast cell tryptase composed mainly of a 31-kDa subunit was found to accumulate in synovial fluid from RA patients and its concentration was higher compared with that from OA patients, although the number of mast cells and amount of mast cell tryptase were similar in synovium from RA and OA patients. Based on these findings, we suggest that a larger amount of mast cell tryptase is released from mast cells into the synovial fluid in RA patients compared with OA patients. This hypothesis is also supported by a recent report [33] that the stimulation of IgE-dependent histamine release from synovial mast cells in RA patients was higher than those in OA patients.

Endoglycosidase H cleaves only high mannose-type sugar chain, while endoglycosidase F cleaves both high mannose-type and complex-type sugar chains from glycoprotein. In this study, endoglycosidase H shifted only the 33-kDa subunit to a position corresponding to the 31-kDa band, and endoglycosidase F decreased the molecular masses of both the 34- and the 33-kDa subunits to 31 kDa. Therefore, the 31-kDa subunit of synovial mast cell tryptase was an unglycosylated form of the 34- and 33-kDa subunits, and the 34- and 33-kDa subunits of mast cell tryptase contain complex-type and high mannose-type sugar chains, respectively. Interestingly, higher concentrations of mast cell tryptase consisting of the unglycosylated subunits were found to accumulate in synovial fluid from RA patients compared with that from OA patients, suggesting enhanced secretion of the unglycosylated mast

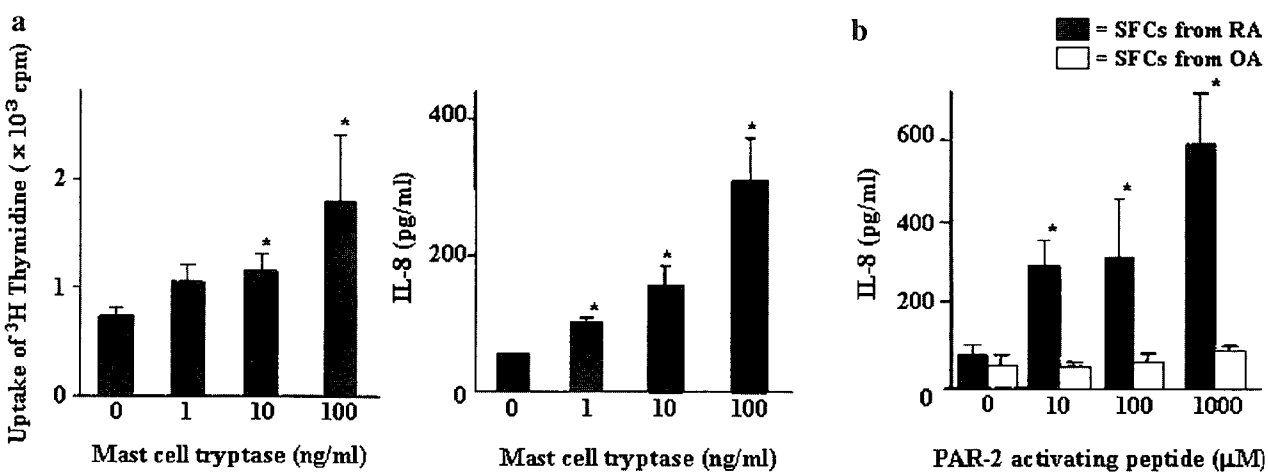

Fig. 4 Effects of human mast cell tryptase and PAR-2 activating peptide on the proliferation of SFCs and IL-8 release from the cells. a SFCs were treated with mast cell tryptase purified from human lung at the indicated concentrations (day 0). On day 1, the proliferation rate of SFCs was estimated by the incorporation of [^{3}H]-thymidine. On day 2 after the treatment, the concentration of IL-8 in media was measured by ELISA. The experiments were conducted in triplicates. The data are the mean (bar denotes SD; [n=3]). *Significantly increased vs no treatment with mast cell tryptase ($P<0.05$). (b) On Day 2 after the treatment with PAR-2 activating peptide at the indicated concentrations, the concentration of IL-8 in media was measured by ELISA. The experiments were conducted in triplicates. The data are the mean (*bar* denotes SD; $n=3$). An *asterisk* denotes a statistically significant increase vs no treatment with mast cell tryptase ($P<0.05$)

cell tryptase into synovial fluids in RA. *N*-Glycosylation has been considered to be necessary for secretion in various secretory proteins [34–36]. However, several lines of investigation have also shown that in the case of prolactin and interferon-γ, the secretion kinetics of the unglycosylated forms was identical to those of the glycosylated forms [37, 38]. Further study is necessary to elucidate the mechanism of increased secretion of mast cell tryptase into synovial fluids in RA. Determining what mediates distinct glycosylation of mast cell tryptase in inflammatory joint diseases is another important subject of investigation.

Ferrell et al. [39] have demonstrated a key role for PAR-2 in mediating chronic inflammation in studies using PAR-2-deficient mice. We have also reported that human trypsin-like protease stimulates human bronchial fibroblast proliferation by a PAR-2-dependent pathway [40]. Therefore, the PAR-2-dependent pathway may be an important component in the pathogenesis of RA, especially with regard the increased production of pro-inflammatory cytokines from synovial cells.

Real-time RT-PCR revealed that expression of PAR-2 mRNA transcripts in the synovium in RA patients was significantly upregulated compared with those from OA patients. We also showed that purified human mast cell tryptase and a PAR-2 activating peptide significantly stimulated IL-8 release in SFCs obtained from RA patients but not from OA patients, indicating that SFCs from RA patients were more sensitive to tryptase than those from OA patients. Increased sensitivity of SFCs may also exacerbate synovitis in RA, in addition to accumulated mast cell tryptase in synovial fluid.

We previously demonstrated that thrombin stimulated the proliferation and IL-8 release in SFCs in the same manner as mast cell tryptase [23]. At present, we cannot determine which proteases play more important roles in the exacerbation of synovitis in RA. However, mast cell tryptase preferentially interacts with PAR-2, while thrombin binds to PAR-1, PAR-3, and PAR-4 [41–43]. Since there was no significant difference in synovial PAR expression between RA and OA except for expression of PAR-2 mRNA transcripts (data not shown), mast cell tryptase may play a more direct role in the proliferation of SFCs and pro-inflammatory cytokine release from these cells compared with thrombin.

This study showed that an unglycosylated form of mast cell tryptase accumulated in higher concentration in the synovial fluid from RA relative to OA and that there was higher expression of synovial PAR-2 mRNA transcripts in RA compared with that in OA. Therefore, higher secretion of mast cell tryptase into synovial fluids in RA relative to OA stimulates higher proliferation of the synovium and production of pro-inflammatory cytokine in SFCs in RA compared with OA.

Acknowledgments We gratefully acknowledged Yukio Higuchi M. D. and Yoshiteru Kawasaki M.D. (Tokushima Prefectural Central Hospital) for collecting the control specimens, and Yuichi Ikeda M.D. and Sachiko Kawano Ph.D. for excellent technical assistance. Financial support for this study was provided by a Grant-in-Aid for Scientific Research (C).

References

1. Malone DG, Wilder RL, Saavedra-Delgado AM, Metcalfe DD (1987) Mast cell numbers in rheumatoid synovial tissues. Correlations with quantitative measures of lymphocytic infiltration and modulation by antiinflammatory therapy. Arthritis Rheum 30:130–137
2. Gruber BL, Schwartz LB, Ramamurthy NS, Irani AM, Marchese MJ (1988) Activation of latent rheumatoid synovial collagenase by human mast cell tryptase. J Immunol 140:3936–3942
3. Bridges AJ, Malone DG, Jicinsky J, Chen M, Ory P, Engber W et al (1991) Human synovial mast cell involvement in rheumatoid arthritis and osteoarthritis. Relationship to disease type, clinical activity, and antirheumatic therapy. Arthritis Rheum 34:1116–1124
4. Dean G, Hoyland JA, Denton J, Donn RP, Freemont AJ (1993) Mast cells in the synovium and synovial fluid in osteoarthritis. Br J Rheumatol 32:671–675
5. Tetlow LC, Woolley DE (1995) Distribution, activation and tryptase/chymase phenotype of mast cells in the rheumatoid lesion. Ann Rheum Dis 54:549–555
6. de Paulis A, Marino I, Ciccarelli A, de Crescenzo G, Concardi M, Verga L et al (1996) Human synovial mast cells. I. Ultrastructural in situ and in vitro immunologic characterization. Arthritis Rheum 39:1222–1233
7. Buckley MG, Gallagher PJ, Walls AF (1998) Mast cell subpopulations in the synovial tissue of patients with osteoarthritis: selective increase in numbers of tryptase-positive, chymase-negative mast cells. J Pathol 186:67–74
8. Gotis-Graham I, Smith MD, Parker A, McNeil HP (1998) Synovial mast cell responses during clinical improvement in early rheumatoid arthritis. Ann Rheum Dis 57:664–671
9. Woolley DE, Tetlow LC (2000) Mast cell activation and its relation to proinflammatory cytokine production in the rheumatoid lesion. Arthritis Res 2:65–74
10. Schwartz LB (1994) Tryptase: a mast cell serine protease. Methods Enzymol 244:88–100
11. Smith TJ, Hougland MW, Johnson DA (1984) Human lung tryptase. Purification and characterization. J Biol Chem 259: 11046–11051
12. Asokananthan N, Graham PT, Fink J, Knight DA, Bakker AJ, McWilliam AS et al (2002) Activation of protease-activated receptor (PAR)-1, PAR-2, and PAR-4 stimulates IL-6, IL-8, and prostaglandin E2 release from human respiratory epithelial cells. J Immunol 168:3577–3585
13. D'Andrea MR, Rogahn CJ, Andrade-Gordon P (2000) Localization of protease-activated receptors-1 and -2 in human mast cells: indications for an amplified mast cell degranulation cascade. Biotech Histochem 75:85–90
14. Smith R, Ransjo M, Tatarczuch L, Song SJ, Pagel C, Morrison JR et al (2004) Activation of protease-activated receptor-2 leads to inhibition of osteoclast differentiation. J Bone Miner Res 19:507–516
15. Steinhoff M, Neisius U, Ikoma A, Fartasch M, Heyer G, Skov PS et al (2003) Proteinase-activated receptor-2 mediates itch: a novel pathway for pruritus in human skin. J Neurosci 23:6176–6180

16. Uehara A, Muramoto K, Takada H, Sugawara S (2003) Neutrophil serine proteinases activate human nonepithelial cells to produce inflammatory cytokines through protease-activated receptor 2. J Immunol 170:5690–5696
17. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS et al (1988) The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 31:315–324
18. Altman R, Asch E, Bloch D, Bole G, Borenstein D, Brandt K et al (1986) Development of criteria for the classification and reporting of osteoarthritis. Classification of osteoarthritis of the knee. Diagnostic and therapeutic criteria committee of the American rheumatism association. Arthritis Rheum 29:1039–1049
19. Nakano S, Ikata T, Kinoshita I, Kanematsu J, Yasuoka S (1999) Characteristics of the protease activity in synovial fluid from patients with rheumatoid arthritis and osteoarthritis. Clin Exp Rheumatol 17:161–170
20. Yasuoka S, Ohnishi T, Kawano S, Tsuchihashi S, Ogawara M, Masuda K et al (1997) Purification, characterization, and localization of a novel trypsin-like protease found in the human airway. Am J Respir Cell Mol Biol 16:300–308
21. Suzue N, Nikawa T, Onishi Y, Yamada C, Hirasaka K, Ogawa T et al (2006) Ubiquitin ligase Cbl-b downregulates bone formation through suppression of IGF-I signaling in osteoblasts during denervation. J Bone Miner Res 21:722–734
22. Nikawa T, Towatari T, Katunuma N (1992) Purification and characterization of cathepsin J from rat liver. Eur J Biochem 204:381–393
23. Mishiro T, Nakano S, Takahara S, Miki M, Nakamura Y, Yasuoka S et al (2004) Relationship between cathepsin B and thrombin in rheumatoid arthritis. J Rheumatol 31:1265–1273
24. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275
25. Cromlish JA, Seidah NG, Marcinkiewicz M, Hamelin J, Johnson DA, Chretien M (1987) Human pituitary tryptase: molecular forms, NH2-terminal sequence, immunocytochemical localization, and specificity with prohormone and fluorogenic substrates. J Biol Chem 262:1363–1373
26. Bradding P, Okayama Y, Howarth PH, Church MK, Holgate ST (1995) Heterogeneity of human mast cells based on cytokine content. J Immunol 155:297–307
27. Little SS, Johnson DA (1995) Human mast cell tryptase isoforms: separation and examination of substrate-specificity differences. Biochem J 307 (Pt 2):341–346
28. Schwartz LB, Sakai K, Bradford TR, Ren S, Zweiman B, Worobec AS et al (1995) The alpha form of human tryptase is the predominant type present in blood at baseline in normal subjects and is elevated in those with systemic mastocytosis. J Clin Invest 96:2702–2710
29. Buckley MG, Walters C, Wong WM, Cawley MI, Ren S, Schwartz LB et al (1997) Mast cell activation in arthritis: detection of alpha- and beta-tryptase, histamine and eosinophil cationic protein in synovial fluid. Clin Sci (Lond) 93:363–370
30. de Paulis A, Ciccarelli A, Marino I, de Crescenzo G, Marino D, Marone G (1997) Human synovial mast cells. II. Heterogeneity of the pharmacologic effects of antiinflammatory and immunosuppressive drugs. Arthritis Rheum 40:469–478
31. Chen Y, Shiota M, Ohuchi M, Towatari T, Tashiro J, Murakami M et al (2000) Mast cell tryptase from pig lungs triggers infection by pneumotropic Sendai and influenza A viruses. Purification and characterization. Eur J Biochem 267:3189–3197
32. Peng Q, McEuen AR, Benyon RC, Walls AF (2003) The heterogeneity of mast cell tryptase from human lung and skin. Eur J Biochem 270:270–283
33. Kiener HP, Baghestanian M, Dominkus M, Walchshofer S, Ghannadan M, Willheim M et al (1998) Expression of the C5a receptor (CD88) on synovial mast cells in patients with rheumatoid arthritis. Arthritis Rheum 41:233–245
34. Orlean P (1992) Enzymes that recognize dolichols participate in three glycosylation pathways and are required for protein secretion. Biochem Cell Biol 70(6):438–447
35. Kukuruzinska MA, Lennon K (1998) Protein N-glycosylation: molecular genetics and functional significance. Crit Rev Oral Biol Med 9:415–448
36. Brodsky JL (2005) An in vitro assay for the selective endoplasmic reticulum associated degradation of an unglycosylated secreted protein. Methods 35:354–359
37. Sareneva T, Pirhonen J, Cantell K, Kalkkinen N, Julkunen I (1994) Role of N-glycosylation in the synthesis, dimerization and secretion of human interferon-gamma. Biochem J 303:831–840
38. Hirooka T, Suganuma N, Furuhashi M, Kitagawa T, Ando H, Kikkawa F et al (1996) Synthesis and release of glycosylated prolactin in transfected cells with human prolactin complementary deoxyribonucleic acid. Endocr J 43:423–428
39. Ferrell WR, Lockhart JC, Kelso EB, Dunning L, Plevin R, Meek SE et al (2003) Essential role for proteinase-activated receptor-2 in arthritis. J Clin Invest 111:35–41
40. Matsushima R, Takahashi A, Nakaya Y, Maezawa H, Miki M, Nakamura Y et al (2006) Human airway trypsin-like protease stimulates human bronchial fibroblast proliferation in a protease-activated receptor-2-dependent pathway. Am J Physiol Lung Cell Mol Physiol 290:L385–L395
41. Dery O, Corvera CU, Steinhoff M, Bunnett NW (1998) Proteinase-activated receptors: novel mechanisms of signaling by serine proteases. Am J Physiol 274:C1429–C1452
42. Hirano F, Kobayashi A, Hirano Y, Nomura Y, Fukawa E, Makino I (2002) Thrombin-induced expression of RANTES mRNA through protease activated receptor-1 in human synovial fibroblasts. Ann Rheum Dis 61:834–837
43. Yang YH, Hall P, Little CB, Fosang AJ, Milenkovski G, Santos L et al (2005) Reduction of arthritis severity in protease-activated receptor-deficient mice. Arthritis Rheum 52:1325–1332

J Neurosurg Spine 7:352–356, 2007

Lumbar radiculopathy caused by extradural rheumatoid nodules

Case report

NORI HIROHASHI, M.D., TOSHINORI SAKAI, M.D., KOICHI SAIRYO, M.D., KOICHI OBA, M.D., KOSAKU HIGASHINO, M.D., SHINSUKE KATOH, M.D., AND NATSUO YASUI, M.D.

Department of Orthopedics, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima, Japan

✓The authors report on a 51-year-old woman with a 9-year history of rheumatoid arthritis (RA) who presented with symptomatic rheumatoid nodules in the lumbar extradural region with compression on the L-5 nerve roots bilaterally. She had also suffered from dysesthesia in the right lower leg and intermittent claudication. Magnetic resonance imaging revealed masses compressing the dural sac, and on lumbar myelography and computed tomography myelography a filling defect at L4–5 was revealed, which was compressing the dural sac posterolaterally on both sides. The masses were surgically removed. On histological examination the typical characteristics of rheumatoid nodules were found. Soon after the operation all of the patient's symptoms disappeared.

There have been few reports on extradural rheumatoid nodules. Patients with RA usually complain of articular symptoms, and in fact the patient in the present study had been referred to the authors' institution for total hip arthroplasty. However, various symptoms other than those arising from articular lesions were found clinically. The authors believe that if patients with RA are also examined for extraarticular lesions, it is likely that these will be more frequently detected. *(DOI: 10.3171/SPI-07/09/352)*

KEY WORDS • **extradural space** • **histological examination** • **lumbar spine** • **rheumatoid arthritis** • **rheumatoid nodule**

RHEUMATOID nodules are extraarticular manifestations of RA, and are one of the seven components of the American Rheumatism Association classification criteria for RA. Rheumatoid nodules generally occur in subcutaneous tissue in regions subjected to mechanical stimulation, such as the elbows and knees. These lesions sometimes occur in other regions, such as the extensor side of the proximal interphalangeal joint, occipital region, and sacrum. It is rare for rheumatoid nodules to cause clinical symptoms and disrupt the activities of daily living in patients with RA. However, if these masses develop in important organs such as the lungs, heart, and meninges, they will disturb daily life activities.[5]

Moreover, there have been few reports in the literature of involvement of the lumbar spine in patients with RA.[1–3,10,11,14] If such patients complain of leg pain, attention is usually focused on articular lesions such as those of the hip and knee joints. However, because there are characteristic lesions of RA in the lumbar spine as well as the cervical spine, attention must also be directed to symptoms originating from the lumbar spine. We report on a patient with symptomatic rheumatoid nodules originating from the lumbar extradural space.

Abbreviations used in this paper: MR = magnetic resonance; RA = rheumatoid arthritis; RF = rheumatoid factor.

Case Report

History and Examination. This 51-year-old woman with a 9-year history of RA had suffered from right hip pain for 2 years. She had been treated medically with prednisolone (10 mg/day) and methotrexate (4 mg/week). She was initially referred to our institution for a total hip arthroplasty because of leg pain. She experienced low back pain, intermittent claudication, and complained of dysesthesia in the right lower leg, which was constant and intensified on sitting. Her sensations, deep tendon reflexes, and the results of manual muscle testing and were normal. Hematological tests revealed a white blood cell count of 8800/mm^3, a C-reactive protein level 0.45 mg/dl, and an RF level of 265 U/ml.

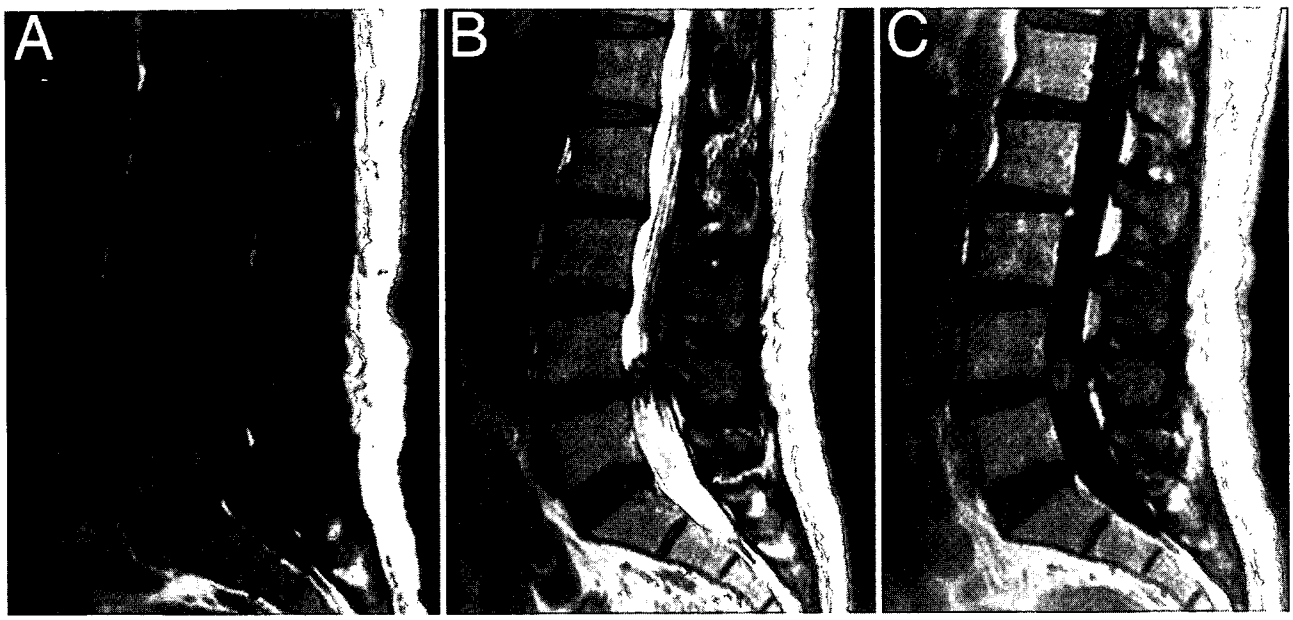

FIG. 1. Sagittal T1-weighted (A), T2-weighted (B), and Gd-enhanced T1-weighted (C) MR images demonstrating the masses in the vertebral canal at L4–5.

Magnetic resonance imaging of the lumbar spine revealed tumorous masses compressing the dural sac at L4–5 that were hypointense on T1-weighted images and hyperintense on T2-weighted images. Magnetic resonance imaging with the addition of contrast medium revealed enhancement around the mass (Figs. 1 and 2). Lumbar and computed tomography myelography revealed a filling defect at the L4–5 level that was compressing the dural sac posterolaterally on both sides (Fig. 3). Based on the imaging results, bilateral facet cysts were initially suspected.

Operation and Histological Findings. Situated within the extradural space, the masses were encapsulated, about 1-cm long, and tightly adherent to the dura mater posterolaterally. The lesions were covered with a yellow membrane and were compressing the dural sac and both nerve roots at L-5 (Fig. 4). Whether a connection existed with the facet joint capsules was unclear, but we found tight adhesion with the dura, suggesting the dura as the origin. The tumorous masses were removed following a partial laminectomy of L-4 and L-5. The bilateral masses could be separated from the dura mater and were carefully resected. After removal of the masses, the dural sac and both L-5 nerve roots were decompressed. Histological examination of the lesions revealed extensive areas of fibrinoid necrosis, surrounded in some parts by poorly formed palisades of histiocytes and chronic inflammatory cells (Fig. 5). These findings were consistent with those of typical rheumatoid granulation tissue.

Postoperative Course. Soon after the operation, the patient's symptoms disappeared, leaving her without neu-

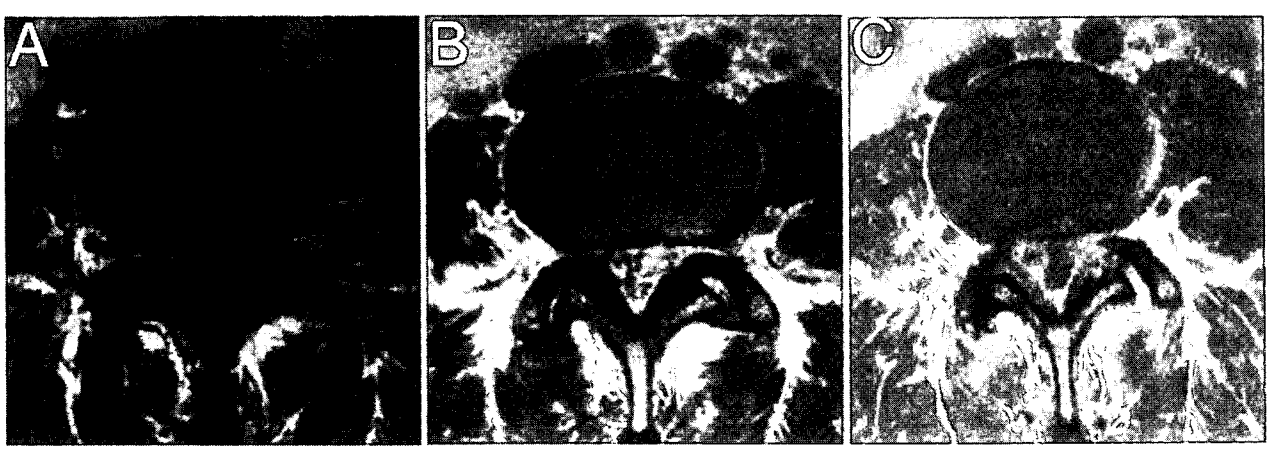

FIG. 2. Axial T1-weighted (A), T2-weighted (B), and Gd-enhanced T1-weighted (C) MR images at L4–5 demonstrating dural sac compression by the masses. Note that the capsule in (C) enhanced with the addition of Gd-diethylenetriaminepentaacetic acid.