J. Biochem. 129, 445–454 (2001)

Apoptotic Cells of an Epithelial Cell Line, AsPC-1, Release Monocyte Chemotactic S19 Ribosomal Protein Dimer[1]

Takumasa Nishimura,*,† Kei Horino,*,† Hiroshi Nishiura,* Yoko Shibuya,‡ Takehisa Hiraoka,† Sumio Tanase,§ and Tetsuro Yamamoto*,[2]

Division of Molecular Pathology, Graduate School of Medical Sciences, and the †First Department of Surgery, the ‡Department of Laboratory Medicine, and the §Second Department of Biochemistry, School of Medicine, Kumamoto University, 2-2-1 Honjo, Kumamoto 860-0811

Received October 27, 2000; accepted December 25, 2000

A pancreatic carcinoma cell line, AsPC-1, underwent apoptosis *in vitro* when heat-treated for 60 min at 43°C. Apoptotic AsPC-1 cells liberated a monocyte chemotactic factor into the culture supernatant 24 to 30 h after the heat-treatment. This factor was immunologically identified as the cross-linked homodimer of S19 ribosomal protein (RP S19), since the majority of the chemotactic activity was absorbed by both anti–RP S19 rabbit antibodies and an anti–isopeptide bond monoclonal antibody immobilized on agarose beads. Intracellular transglutaminase activity increased during the apoptotic process, reaching the peak strength between 18 and 24 h after the heat-treatment. A recombinant RP S19 acquired the monocyte chemotactic capacity when incubated with the apoptotic cell extract obtained at the 18th hour. The chemotactic activity acquirement as well as the transglutaminase activity were blocked by treatment of the extract with anti–type II transglutaminase rabbit antibodies. When the recombinant RP S19 was treated with an authentic type II transglutaminase, the dimerization of RP S19 concomitant with the generation of the monocyte chemotactic activity was observed. Peptide-map analyses involving amino acid sequencing demonstrated that the inter-molecular isopeptide bond was heterogenous: Gln12 or Gln137 and Lys29 or Lys122 were cross-linked. Site-directed mutagenic analysis indicated that the cross-linking of Gln137, but not other residues such as Gln12, Lys29, and Lys122, was essential for expression of the chemotactic activity.

Key words: apoptosis, chemotactic factor, isopeptide bond, monocytes, S19 ribosomal protein, transglutaminase.

S19 ribosomal protein (RP S19) is a component of the small subunit of ribosomes that comprise the protein-producing machinery. Interestingly, RP S19 has an extra-ribosomal function: when it becomes a cross-linked homodimer through a transglutaminase-catalyzed reaction, it gains monocyte chemotactic activity. The optimum concentration, 10^{-9} M, of the RP S19 dimer in the chemotaxis chamber assay is comparable to those of other chemotactic factors such as C5a (the complement C5–derived chemotactic peptide), formyl-Met-Leu-Phe (a bacteria-derived chemotactic peptide), and monocyte chemoattractant protein–1 (a chemokine) (*1*). The RP S19 dimer attracts monocytes by means of binding to their C5a receptor due to molecular mimicry as to C5a, although the calculated homology of the amino acid sequences of RP S19 and C5a is only 4% (*1*).

Natural formation of the RP S19 dimer with the monocyte chemotactic capacity was first demonstrated at rheumatoid arthritis synovial lesions as the major monocyte chemotactic factor (*2*). Later we demonstrated that the RP S19 dimer was formed and released during the apoptotic process using the HL-60 cell line derived from a human monocytic leukemia cells. We also revealed the biological role of the RP S19 dimer through an experiment involving the intradermal injection of apoptotic HL-60 cells. The liberation of the RP S19 dimer resulted in the recruitment of circulating monocytes to the apoptotic cells followed by phagocytic clearance of the apoptotic cells by the infiltrated monocytes/macrophages (*3*).

Because of their derivation, HL-60 cells possess the A subunit of factor XIII, which is the zymogen of the plasma transglutaminase, factor XIIIa. This led to our previous study, in which factor XIIIa was used *in vitro* to form the dimer of recombinant RP S19 molecules (*4*). Although factor XIIIa catalyzed the dimerization of RP S19 with concomitant expression of the chemotactic activity, the enzymatic reaction required heparin as a cofactor. That the factor XIII A subunit is only produced by monocytes/macrophages and megakaryocytes raised the question of whether the RP S19 dimer formation during apoptosis is specific for

[1] This research was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (T.Y., 12470058).

[2] To whom correspondence should be addressed. Phone: +81-96-373-5304, Fax: +81-96-373-5308, e-mail: tetsu@gpo.kumamoto-u.ac.jp

Abbreviations: RP S19, S19 ribosomal protein; HBSS, Hank's balanced salt solution; ZAP, zymosan-activated plasma; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP-digoxigenin nicked end labeling; PMN, polymorphonuclear leukocytes; TFA, trifluoroacetic acid; NEM, *N*-ethylmaleimide; DFP, diisopropyl fluorophosphate.

the leukocyte-lineage cells or universal for all nucleated cells possessing ribosomes with RP S19. Most cells including HL-60 cells possess type II transglutaminase (tissue transglutaminase), and type II transglutaminase is the transglutaminase that increases during the apoptotic process (*5*).

The current study was carried out to answer this question. We used the AsPC-1 cell line, which is derived from a pancreatic cancer cell, as a representative of epithelial cells that possess type II transglutaminase but not the factor XIII A subunit. Type II transglutaminase effectively cross-linked RP S19 without the aid of heparin.

EXPERIMENTAL PROCEDURES

Materials and Reference Compounds—Hank's balanced salt solution (HBSS) and RPMI1640 medium were purchased from Nissui Pharmaceutical (Tokyo). BSA and TPCK-trypsin were purchased from Sigma (St. Louis, MO). Fetal bovine serum was obtained from Nippon B.M.A. (Tokyo). ApopTag® was a product of Oncor (Gaithersburg, MD). Type II transglutaminase purified from guinea pig liver was purchased from Oriental Yeast Co. (Osaka, Japan). The transglutaminase assay kit (Iatro-FL FXIIIR®) was a product of Iatron Laboratories (Tokyo). An electrophoresis calibration kit for molecular weight determination of low molecular weight proteins was purchased from Pharmacia (Uppsala, Sweden). All other chemicals were obtained from Wako Pure Chemicals (Osaka), unless otherwise specified. A multiwell chamber for the chemotaxis assay was obtained from Neuro Probe (Bethesda, MD). Polycarbonate filters for the multiwell chamber were purchased from Nuclepore (Pleasanton, CA). An AM-302 (ODS; C18) column was purchased from YMC Company (Kyoto). Zymosan-activated plasma (ZAP) was prepared according to the method of Fernandez *et al.* (*6*) with a modification, as described previously (*7*).

Anti–type II transglutaminase rabbit serum was kindly provided by Prof. Koji Ikura (Kyoto Institute of Technology, Kyoto). Anti–factor XIII A subunit rabbit serum was a product of Calbiochem (La Jolla, CA). Horseradish peroxidase (HRP)–labeled anti–rabbit IgG goat IgG was a product of Zymed Laboratories (South San Francisco, CA). Anti–isopeptide bond monoclonal antibody beads were purchased from Coval Ab (Oullins Cedex, France). Sepharose 4B beads bearing monospecific chicken antibodies against RP S19 or the control chicken IgG were prepared as described previously (*3*).

Cell Culture and Induction of Apoptosis—Human pancreatic adenocarcinoma cell line AsPC-1 was purchased from Dainippon Pharmaceutical (Osaka). To grow the cells, they were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine. AsPC-1 cells could not be kept in a serum-free defined medium such as Cosmedium 001.

To induce apoptosis, AsPC-1 cells (5×10^5 cells/ml) were heated for 60 min at 43°C in a water bath, and then kept at 37°C in a CO_2 incubator. Aliquots of these AsPC-1 cells in the medium were taken at 1, 6, 12, 18, 24, 30, and 36 h after the heat treatment. As a control, AsPC-1 cells were treated in the same way except at 37°C instead of 43°C. Each aliquot was subjected to successive centrifugation at 400 ×*g* for 5 min and at 20,000 ×*g* for 20 min at 4°C, and the supernatant obtained was used for the chemotaxis assay. The precipitated cell fraction was used for the preparation of a cytoplasmic extract.

Cytological Examination of Apoptosis—Apoptosis of AsPC-1 cells was detected by the terminal deoxynucleotidyltransferase-mediated dUTP-digoxigenin nicked end labeling (TUNEL) method using the ApopTag® kit. In brief, AsPC-1 cells were treated for 60 min at 43°C, and aliquots were collected at 6 h intervals for 36 h. The latter were centrifuged at 1,000 rpm for 5 min and then the cell density was adjusted to approximately 5×10^7 cells/ml, followed by fixing in 4% phosphate-buffered formalin for 10 minutes at room temperature. Smears of the samples were prepared and dried for 30 min at room temperature. Endogenous peroxidase was quenched with 3% hydrogen peroxide in PBS for 5 min at room temperature. The following process was carried out according to the instruction manual for the kit.

Immunoadsorption with Antibody Beads—For an immunoadsorption experiment with the anti–RP S19 chicken antibodies, a batch-wise method was used. Briefly, the 24 h culture supernatant (200 μl) was incubated with the anti–RP S19 chicken IgG beads (200 μl) for 30 min at 22°C with continuous shaking. After centrifugation at 10,000 ×*g* for 20 min at 4°C, the supernatant was recovered and then polarization activity remaining in it was measured. In an other absorption experiment with the anti–isopeptide bond monoclonal antibody, immunoaffinity column chromatography was performed. The details of this method are given below ("*Preparation of Dimeric Recombinant RP S19*").

Chemotaxis Assay—Monocytes and polymorphonuclear leukocytes (PMN) were isolated from heparinized human peripheral venous blood of healthy donors according to the method of Fernandez *et al.* (*6*) as described previously (*7*, *8*). The monocytes and PMN were suspended at a cell density of 1×10^6 cells/ml in HBSS containing 0.5% bovine serum albumin, pH 7.4, for the morphologic polarization assay. The morphologic polarization assay was performed according to the method of Cianciolo and Snyderman (*9*), as described previously (*7*, *8*). Unless otherwise specified, the assay samples were diluted at least 10-fold with HBSS prior to the assay. As positive and negative controls, 1% (v/v) ZAP and HBSS were used as chemoattractants, respectively. The activity of the samples was initially calculated as the percentage of monocytes or PMN with a polarized morphology compared with the total numbers of cells counted. The percentage of polarized cells was proportional to the logarithmic change in the concentration of samples or ZAP when the concentration was low (below 10 %). Polarization activity was converted and expressed as an arbitrary unit; one polarization unit corresponded to the activity contained in 1% ZAP (*7*, *8*). In the multiwell chamber assay, the monocytes and PMN were respectively suspended at a concentration of 1×10^6 cells/ml in RPMI1640 containing 10% fetal bovine serum and in HBSS containing 0.5% BSA (pH 7.2). The multiwell chamber was used according to the method of Falk *et al.* (*10*) using a nuclepore filter with a pore size of 3 μm for PMN or of 5 μm for monocytes, respectively. After incubation for 90 min, each membrane was separated, fixed with methanol, and stained with Giemsa. The total number of monocytes and PMN migrating beyond the lower surface of the membrane was

determined in five high-power fields. The results were expressed as the numbers of migrating leukocytes.

Separation and Characterization of the Monocyte Chemotactic Factor in an Apoptotic Cell Culture Supernatant—At 24 h after heat treatment, a cell culture was treated with a mixture of protease inhibitors: 0.5 mM *N*-ethyl maleimide (NEM), 2 mM EDTA, and 1 mM diisopropyl fluorophosphate (DFP), for 5 min at room temperature, and centrifuged at 400 ×*g* for 5 min at room temperature to obtain a culture supernatant. This supernatant was centrifuged again at 20,000 ×*g* for 20 minutes at 4°C.

Preparation of the Wild Type and Mutant Types of Recombinant RP S19 Molecules—Seven types of recombinant RP S19, *i.e.* the wild type and six mutants (Lys29Gly-RP S19, Lys122Gly-RP S19, Gln11Ser-Gln12Ser-RP S19, Gln137Asn-RP S19, Lys29Gly-Lys122Gly-RP S19, and Gln11Ser-Gln12Ser-Gln137Asn-RP S19), were prepared as described previously (*2*). The method used for the preparation of the mutant RP S19 recombinant plasmids (pET11a; Novagen, Madison, WI) was polymerase chain reaction–mediated site-directed mutagenesis (*11*). Each construct of the wild type and mutant RP S19 recombinant plasmids was transformed to the expression host, *E. coli* BL21(DE3) competent cells (Novagen).

These cells were stimulated to synthesize recombinant RP S19 with isopropyl beta-D-thiogalactoside. The wild type and mutant recombinant RP S19 molecules extracted from the periplasmic fraction of the *E. coli* were purified by HPLC on an SP-5PW column (Tosoh Company, Tokyo) and a HiTrap heparin column (Pharmacia), in that order, as described previously (*4*). Judging from the SDS-PAGE patterns, their purity was always more than 95%. The protein concentrations of the wild type and mutant recombinant RP S19 molecules were determined as the absorbance at 280 nm under the assumption that the absorbance unit, 1.0, was equivalent to 1 mg/ml.

Preparation of Dimeric Recombinant RP S19—Recombinant RP S19 molecules were incubated for 60 min at 37°C in the presence of 0.5 unit/ml of type II transglutaminase, 5 mM $CaCl_2$ and 1 mM DTT. The product was applied to an anti–isopeptide bond monoclonal antibody column (∅ 10 × 25 mm, 2 ml bed volume) equilibrated with 10 mM Tris-HCl containing 100 mM NaCl (pH 7.5). After the breakthrough fraction had been recovered, the column was washed with the equilibration buffer. Then, proteins bound to the column were eluted with 10 mM Tris-HCl containing 1 M NaCl (pH 7.5). The sample eluted with 1 M NaCl was mixed with trifluoroacetic acid (TFA) and acetonitrile at final concentrations of 0.1 and 5%, respectively, and then subjected to reverse phase HPLC on a C4 column (Hitachi 5C4-300, Nacalai Tesque, Kyoto) equilibrated with 0.1% TFA containing 5% acetonitrile. The recombinant RP S19–derived molecules were eluted with a shallow gradient change of the acetonitrile concentration.

Measurement of Intracellular Transglutaminase Activity—The extraction procedure used for intracellular transglutaminase was a modification of the method of Cheng and Chung (*12*). One ml of extract was obtained from 1.5 × 10^6 cells. The transglutaminase activity was measured by a modification of the method of Lorand *et al.* (*13*) using an assay kit, Iatro-FL F XIIIR®. When the sensitivity of the intracellular transglutaminase activity to the anti–type II transglutaminase rabbit antibodies was examined, an extract of AsPC-1 cells prepared at 18 hours after heat-treatment was mixed with the rabbit antiserum euglobulin fraction at a ratio of 9 to 1 in volume, and then incubated for 60 min at 37°C. Three different protein concentrations of the antiserum, such as 5, 50, and 500 μg/ml, were used as the final concentration. After high-speed centrifugation, the supernatants obtained were subjected to the enzyme assay.

Treatment of RP S19 with an Apoptotic Cell Extract—The wild type RP S19 (final concentration, 230 μg/ml) was incubated for 60 min at 37°C with an apopptotic AsPC-1 cell extract obtained at 18 h after heat-treatment in the presence of 10 mM $CaCl_2$. The final concentration of the extract in the incubation mixture was 45% of the initial extract of 1.5 × 10^6 cells/ml, and it contained a concentration of about 0.15 U/ml transglutaminase as enzyme activity. In some experiments, the cell extract was pretreated with the anti–type II transglutaminase rabbit antiserum euglobulin fraction (final concentration, 50 μg/ml) or with an equivalent normal rabbit fraction for 60 minutes at 37°C. After high-speed centrifugation, the supernatants obtained were respectively used for the treatment of RP S19.

SDS-PAGE—Electrophoresis was performed on a vertical slab gel of 15% polyacrylamide according to the method of Laemmli (*14*). The sample was boiled for 2 min in the presence of SDS and then applied to the gel. After electrophoresis at 20 mA for 40 min, the gel was stained with Coomassie Brilliant Blue.

Determination of Cross-Linked Residues by Peptide Mapping and Amino Acid Sequencing Analyses—Peptide mapping and amino acid sequencing analyses were performed as described previously (*4*). In brief, the monomer and cross-linked dimer were respectively treated with TPCK-trypsin for 24 h at 37°C. The fragmented peptides were subjected to reverse phase HPLC on a C18 column which had been equilibrated with 0.1% TFA containing 5% acetonitrile, and eluted from the column with a gradient change in the acetonitrile concentration from 5 to 80%. After evaporation in a vacuum centrifuge concentrator, limited amino terminal amino acid sequencing was performed in duplicate for 20 cycles with a protein sequencer (Applied Biosystems, 477A) equipped with a PTH amino acid analyzer (Applied Biosystems 120A) according to the instruction manual.

RESULTS

Induction of Apoptosis in AsPC-1 Cells by Hyperthermia—Apoptosis was induced in AsPC-1 cells by heat-treatment for 60 min at 43°C. When the immuno-histochemical detection of the DNA fragmentation was performed according to the method of Gavrieli *et al.* (*15*) (TUNEL method), the ratio of apoptotic cells became more than 80% and 90% at 24 h and 30 h after the heat-treatment, respectively (Fig. 1). A heat-treatment period of 60 min was required to obtain a high apoptotic ratio. With the same treatment for 30 min, less than 50% of the cells underwent apoptosis (data not shown).

Liberation of the Monocyte Chemotactic Factor into the Culture Supernatant of Apoptotic AsPC-1 Cells—As shown in Fig. 2, monocyte chemotactic activity appeared in the culture supernatant of AsPC-1 cells, peaking at 30 h after heat-treatment for 60 min at 43°C. In a certain batch of cultures, the peak was observed at 24 h (one interval earlier in

the observation period) after the treatment. On the other hand, no chemotactic activity was observed in the control culture supernatant throughout the observation period of 36 h. These phenomena are almost identical to those previously observed in experiments involving HL-60 cells (*3*), except for delay for AsPC-1 of 6 h in the process. The chemotactic activity toward PMN of the 30 h culture supernatants obtained from the heat-treated cells was negligible (less than 1/1,000 of that of ZAP) (data not shown). The monocyte-restricted nature suggested that the major

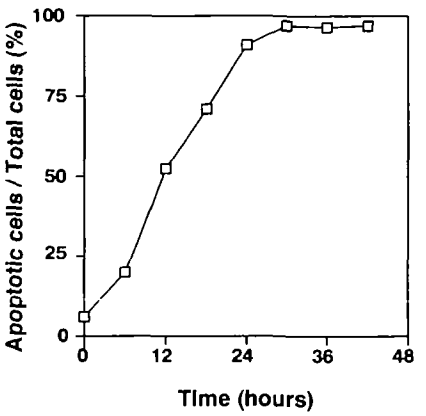

Fig. 1. **Apoptosis induction in AsPC-1 cells by hyperthermia.** Cultured cell suspensions with a density of 5×10^5 cells/ml were heat-treated for 60 min at 43°C and then cultured again. Aliquots of the cultured cell suspensions were taken and subjected to cytochemical DNA fragmentation analysis with the TUNEL method. TUNEL-positive and -negative cells were counted at random till a total of 100 cells, and then the ratio of positive cells to the total was calculated for each sample. The ratio of the TUNEL-positive cells as a percentage was plotted as a function of time.

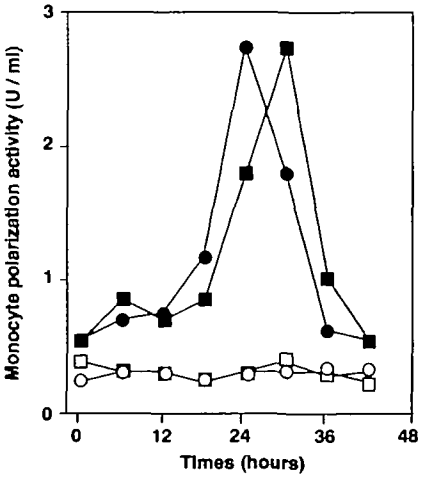

Fig. 2. **Generation of monocyte chemotactic factor by AsPC-1 cells during apoptosis.** AsPC-1 cells (5×10^5 cells/ml) were heat-treated for 60 min at 43°C (closed symbols) or kept at 37°C as a control (open symbols). At various times after the heat-treatment, aliquots of the culture cell suspension were taken. After centrifugation, the monocyte chemotactic activity in the culture supernatant was measured by means of the polarization assay. Time 0 indicates just before the heat-treatment. The results of two separate experiments are shown by circles (experimental 1) and squares (experiment 2), respectively.

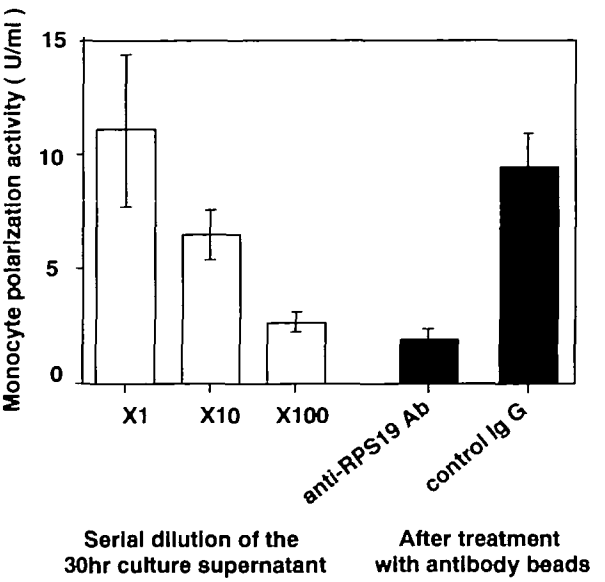

Fig. 3. **Immunoabsorption of monocyte chemotactic activity in the apoptotic cell culture supernatant with anti-RP S19 antibody beads.** An aliquot (500 μl) of the culture supernatant of heat-treated AsPC-1 cells at 24 h (Fig. 2, experiment 1) was pre-treated with protease inhibitors for 5 min at room temperature. The sample was then immunologically treated with 100 μl of immunobeads on which either anti-RP S19 chicken IgG or normal chicken IgG had been immobilized. After the immunologic treatment for 30 min at room temperature, the supernatants were recovered and the monocyte polarization activity in them was measured in triplicate (closed columns). For brief quantitation of the absorption efficacy, an aliquot of the pre-treated sample (24 hour culture supernatant) was serially diluted, and then the monocyte chemotactic activity was measured (open columns).

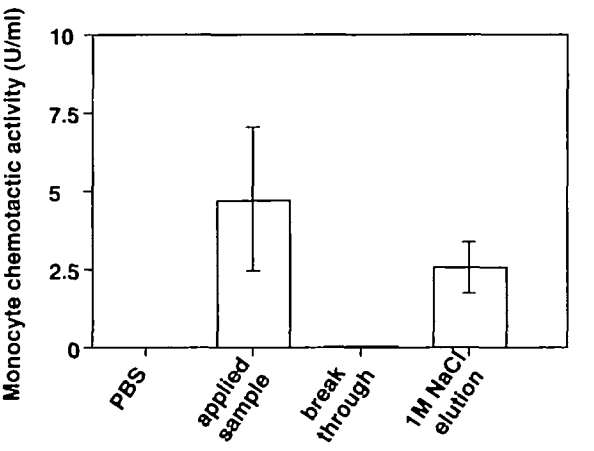

Fig. 4. **Affinity column chromatography with anti-isopeptide bond antibody beads.** An aliquot (2 ml) of the culture supernatant obtained at 24 h after the heat-treatment was pretreated with protease inhibitors for 5 min at room temperature and then applied to an immuno affinity column of anti-isopeptide bond monoclonal antibody beads, which had been equilibrated with 10 mM Tris-HCl buffer containing 120 mM NaCl (pH 7.4), at the flow rate of 0.5 ml/min at 4°C. Bound molecules were eluted by a stepwise salt elution with 10 mM Tris-HCl buffer containing 1M NaCl. The monocyte polarization activity was measured in triplicate.

chemotactic factor in the culture supernatants would be the cross-linked homodimer of RP S19.

Immunologic Identification of the Monocyte Chemotactic Factor in the Apoptotic Cell Culture Supernatant at 30 h—When the supernatant obtained at 24 h after the heat-treatment was treated with the anti-RP S19 chicken antibody beads by the batch-wise method, the majority of the monocyte chemotactic capacity was adsorbed on the beads (Fig. 3). The remaining unbound chemotactic activity was almost equivalent to that of a 100-fold dilution of the initial supernatant. This result was reproducible in two separate experiments.

Because the chemotactic RP S19 dimer is cross-linked *via* an isopeptide bond, this dimer should be absorbed by the anti-isopeptide bond antibody beads. We applied the 24 h culture supernatant to a column packed with the anti-isopeptide bond antibody beads. As shown in Fig. 4, all the monocyte chemotactic capacity bound to the column, and a significant part of it was eluted from the column with 10 mM Tris-HCl containing 1 M NaCl (pH 7.5). This result was also reproducible in two separate experiments.

The immunological data indicate that the RP S19 dimer accounts for the majority of the monocyte chemotactic activity in the culture supernatant of the apoptotic AsPC-1 cells.

Immuno-Cytological Observation of Type II Transglutaminase in AsPC-1 Cells—AsPC-1 cells were immuno-cytochemically stained for type II transglutaminase and for the factor XIII A subunit before and 30 h after the heat-treatment to induce apoptosis. The avidin-biotin method involving a Pathostain ABC-POD® kit (Wako) was used to visualize the immune complexes. In terms of type II transglutaminase, immuno-reactivity was present in the cytosol of AsPC-1 cells at both times. The intensity of the staining was stronger at 30 h after the heat-treatment than before

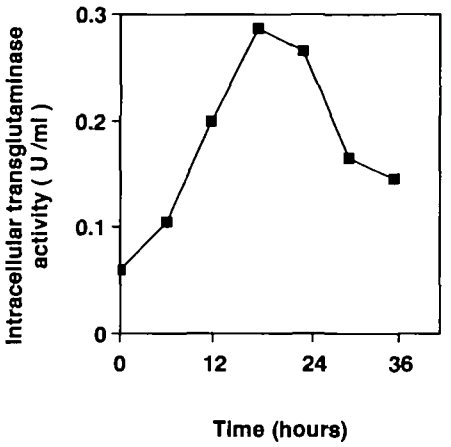

Fig. 5. **Chronological change in the intracellular transglutaminase activity during apoptosis of AsPC-1 cells.** AsPC-1 cells (5×10^5 cells/ml) were heat-treated for 60 min at 43°C. At various times after the heat-treatment, aliquots of the culture cell suspension were taken. After centrifugation, the precipitated cell fraction was resuspended in the extraction buffer at the cell density of 1.5×10^6 cell/ml and then treated with a Polytoron® homogenizer for 3 min on ice. After extraction for 60 min on ice, the samples were centrifuged, and the supernatants were used for the measurement of transglutaminase activity with an assay kit. One unit was defined as the activity contained in 1 ml of normal human plasma as factor XIII (zymogen of plasma transglutaminase).

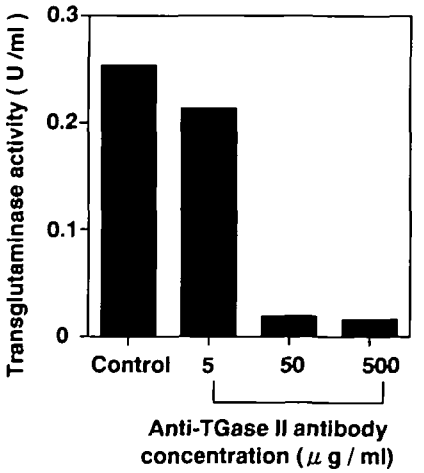

Fig. 6. **Effect of anti-type II transglutaminase antibodies on enhanced transglutaminase activity during apoptosis of AsPC-1 cells.** The extract of AsPC-1 cells prepared at 18 h after the heat-treatment shown in Fig. 5 was mixed with the rabbit antiserum euglobulin fraction at the final concentration of 5, 50, or 500 μg/ml, followed by incubation for 60 min at 37°C. For the control, the vehicle buffer used for the antiserum solution was used. After high-speed centrifugation, the supernatants were subjected to the transglutaminase enzyme assay. TGase II means type II transglutaminase.

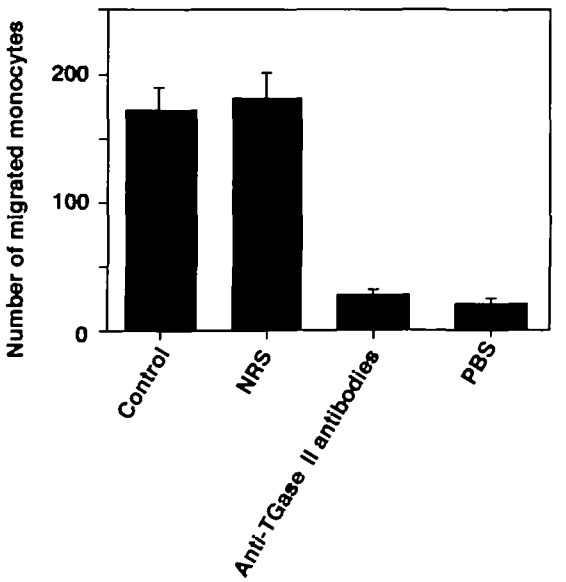

Fig. 7. **Type II transglutaminase-dependent generation of monocyte chemotactic activity on the treatment of recombinant RP S19 with the apoptotic AsPC-1 cell extract.** A wild type recombinant RP S19 was treated for 60 min at 37°C with the 18 h apoptotic cell extract used in Fig. 6 in the presence of 5 mM $CaCl_2$ and 1 mM DTT, and then the monocyte chemotactic activity was measured (Control). Neither this extract nor the recombinant RP S19 possessed chemotactic activity. In some experiments, this extract was pretreated for 60 min at 37°C with the anti-type II transglutaminase rabbit antiserum euglobulin fraction (Anti-TGase II antibodies) or normal rabbit serum euglobulin fraction (NRS) at the final concentration of 50 μg/ml. PBS denotes phosphate-buffered saline (pH 7.4) used as a negative control in the monocyte chemotaxis assay.

the treatment. In contrast to this, anti–factor XIII A subunit antibodies did not demonstrate a positive reaction (data not shown). These results indicated that AsPC-1 cells possess type II transglutaminase but not factor XIIIa, and that the amount of type II transglutaminase seemed to increase during the apoptotic process.

Chronological Change of Type II Transglutaminase Activity in AsPC-1 Cells during Apoptosis—Enzymatic measurement of the intracellular transglutaminase level was carried out using extracts of AsPC-1 cells obtained at various times after the heat-treatment. As shown in Fig. 5, the intracellular transglutaminase activity increased during the apoptotic process, peaking between 18 and 24 h. The majority (93%) of the transglutaminase activity in the extract at 18th hour was suppressed by treatment with the anti–type II transglutaminase antibodies at the final concentration 50 μg/ml (Fig. 6).

Type II Transglutaminase–Dependent Generation of Monocyte Chemotactic Activity on Treatment of Recombinant RP S19 with the Apoptotic AsPC-1 Cell Extract—To examine the participation of type II transglutaminase in the generation of the monocyte chemotactic RP S19 dimer during the apoptotic process, the wild type recombinant RP S19 was treated with the apoptotic cell extract obtained after 18 h for 60 min at 37°C. Neither this extract nor the recombinant RP S19 possessed the chemotactic activity. However, the monocyte chemotactic activity appeared after this treatment. When this extract was pretreated with the anti–type II transglutaminase antibodies (euglobulin fraction of rabbit antiserum), the capacity of the extract to generate the chemotactic activity in the RP S19 preparation was almost totally lost. In contrast to this, the capacity of the extract remained after the pretreatment with normal rabbit serum euglobulin fraction (Fig. 7).

These results strongly suggested that type II transglutaminase in the apoptotic cells catalyzed the cross-linked dimerization of RP S19 accompanied by expression of the chemotactic activity. To elucidate the details of the type II transglutaminase-catalyzed dimerization reaction of RP S19, we used an authentic type II transglutaminase in the following experiments.

Cross-Linked Dimerization of RP S19 with Type II Transglutaminase In Vitro—The recombinant RP S19 (3.0 mg/ml) was incubated for 60 min at 37°C with type II transglutaminase (0.5 unit/ml) in the presence of 5 mM $CaCl_2$ and 1 mM DTT, and the reaction products were analyzed by SDS-PAGE. Since the cross-linking of RP S19 with factor XIIIa was enhanced in the presence of heparin (*4*), the effect of heparin on the reaction with type II transglutaminase was also examined. As shown in Fig. 8, the dimer and tetramer of RP S19 were formed within the incubation period of 60 min. The efficacy of the RP S19 cross-linking in the absence of heparin was much higher than that with factor XIIIa (*4*). The apparent efficacy of the cross-linking by type II transglutaminase seemed equivalent to that by factor XIIIa under the optimal conditions with 1 unit/ml heparin. Different from the case with factor XIIIa, heparin did not augment the cross-linking. Instead, even the presence of 0.01 unit/ml of heparin interfered with the reaction. This interference seemed to occur due to aggregation of RP S19 and heparin molecules, since the reaction mixture became turbid when heparin was added.

The cross-linked dimer was separated by HPLC, and its monocyte chemotactic capacity was measured by means of

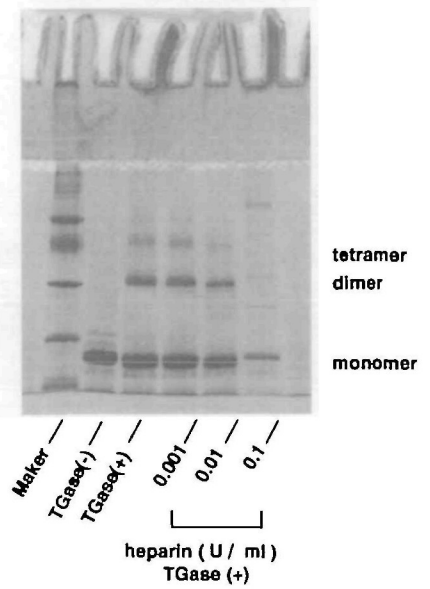

Fig. 8. **RP S19 dimerization by type II transglutaminase and the effect of heparin on it.** In the absence or presence of various concentrations of heparin (0.001 unit/ml to 0.1 unit/ml), the recombinant RP S19 (2.0 mg/ml) in 10 mM Tris-HCl buffer containing 100 mM NaCl and 5 mM $CaCl_2$ (pH 7.5) was incubated with type II tissue transglutaminase (0.5 unit/ml) for 60 min at 37°C, and then the products were analyzed by SDS-PAGE (15% gel, Coomassie Brilliant Blue staining). In the left most lane, molecular weight marker proteins were ran (phosphorylase *b*, MW 94 k; bovine serum albumin, MW 67 k; ovalbumin, MW 43 k; carbonic anhydrase, MW 30 k; soybean trypsin inhibitor, MW 20 k; and alpha-lactalbumin, MW 14 k).

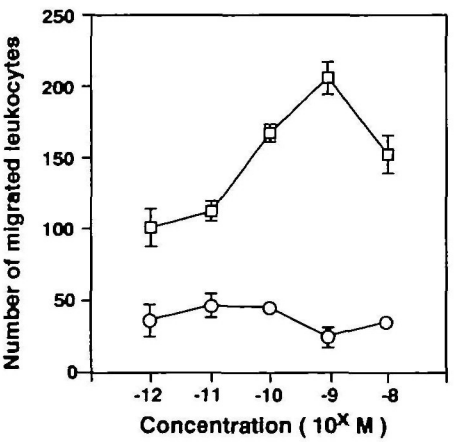

Fig. 9. **Leukocyte chemotactic capacities of the RP S19 dimer cross-linked with type II transglutaminase.** Various concentrations, from 10^{-12} M to 10^{-8} M, of the RP S19 dimer cross-linked with type II transglutaminase in the absence of heparin were subjected to the chemotactic assay using a multiwell chamber and a Nuclepore filter with a pore size of 3 μm or 5 μm for polymorphonuclear leukocytes (PMN) or monocytes, respectively. After 90 min incubation, the membrane was stained with Giemsa solution, and then the number of monocytes (open squares) or PMN (open circles) that had migrated beyond the membrane was determined in five high-power fields. Values are expressed as means ± SD (n = 3 for each experiment).

the chamber assay. As shown in Fig. 9, strong chemotactic activity for monocytes but not for PMN was observed. The dose-function relationship of the monocyte chemotactic activity exhibited a bell-shape pattern, the optimal concentration being 10^{-9} M.

Analysis of Cross-Linked Gln and Lys Residues by Peptide-Mapping and Amino Acid Sequencing—The untreated monomer and cross-linked dimer of the recombinant RP S19 were respectively treated with TPCK-trypsin, and then subjected to reverse phase HPLC on a C18 column to prepare their trypsin-digested peptide maps. Their chromatographic patterns are comparatively shown in Fig. 10. The peptide peaks which are significantly different between the monomer and dimer are indicated, with their amino acid sequences, in the figures. It was demonstrated that the peptides containing either Gln12, Gln137, Lys29, or Lys122, such as DVNQQEFVR, IAGQVAAANK, GGAGVGSMTK and KLTPQGQR, disappeared, as RP S19 is cross-linked by type II transglutaminase. Inversely, dimer peptides such as IAGX(Q)VAAANK/X(K)LTPQGQR, DVNQX(Q)EFVR/X(K)LTPQGQR, and DVNQX(Q)EFVR/GGAGVGSMTX(K) newly appeared. These results indicated that most of the dimer was cross-linked between either Gln12 and Lys29, Gln12 and Lys122, or Gln137 and Lys122.

Analysis of Cross-Linked Residues Using Mutagenic Recombinant RP S19 Molecules—To confirm the interchangeability between Gln12 and Gln137, and that between Lys29 and Lys122 of RP S19 as the substrate residues for type II transglutaminase, four RP S19 mutants in which one of these residues was substituted by another amino acid residue, and two mutants in which both the Gln residues or Lys residues were substituted were prepared. In the case of the Gln12 mutant, Gln11 was also substituted by Ser, because the Gln residues in the double Gln sequence are sometimes mutually interchangeable (*16*). When Gln11Ser-Gln12Ser-RP S19, Gln137Asn-RP S19, Lys29Gly-RP S19, and Lys122Gly-RP S19 were respectively treated with type II transglutaminase for 60 min at 37°C, significant amounts of dimer molecules were produced in all cases. As shown in Fig. 11 (a and b), the amounts of the dimers were comparable to in the case of the wild type RP S19 treatment. In contrast to this, when Gln11Ser-Gln12Ser-Gln137Asn-RP S19 and Lys29Gly-Lys122Gly-RP S19 were treated in the same way, no dimerization of them was observed (Fig. 11c).

The monocyte chemotactic capacities were then compared among the dimers and monomers of the wild type and mutants of the recombinant RP S19. The chemotactic capacities were also compared between the dimer and monomer of each kind of recombinant RP S19. The results are shown in Fig. 12. Consistently with no dimerization on SDS-PAGE analysis, Gln11Ser-Gln12Ser-Gln137Asn-RP S19 did not express the monocyte chemotactic activity even after treatment with the transglutaminase. The dimers of Gln11Ser-Gln12Ser-RP S19, Lys29Gly-RP S19, and Lys122Gly-RP S19 exhibited as much monocyte chemotactic capacity as the dimer of the wild type RP S19, indicating that the cross-linking at either Gln12, Lys29 or Lys122 is not related to expression of the monocyte chemotactic activity. In contrast to this, the Gln137Asn-RP S19 dimer exhibited significantly lower capacity. Furthermore, in contrast to the monomers of the wild type, Gln11Ser-Gln12Ser, Lys-

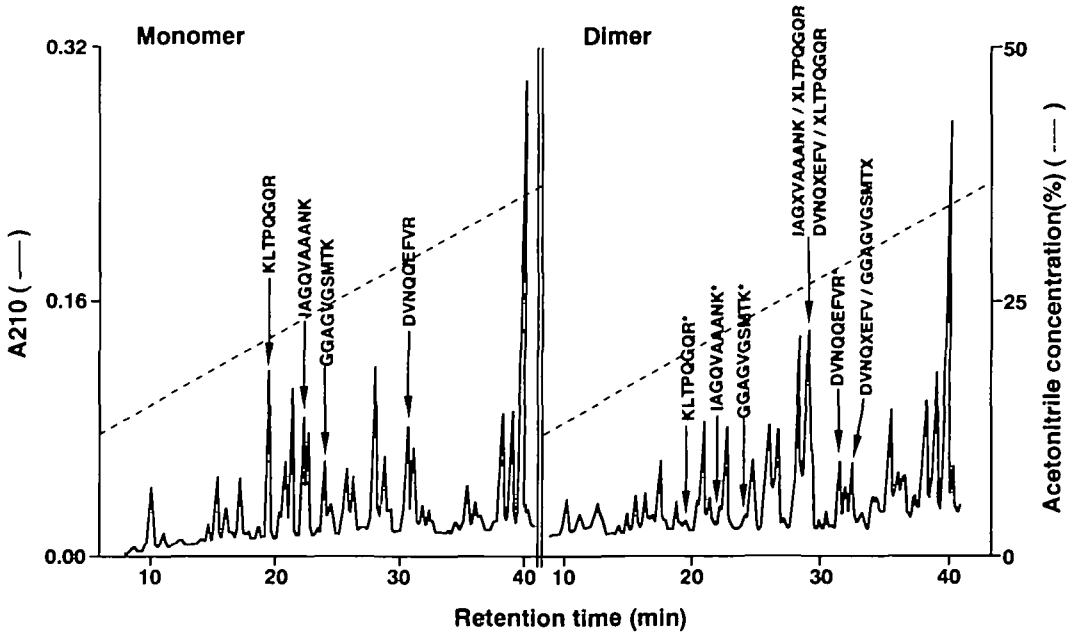

Fig. 10. **Examination of Gln and Lys residues of RP S19 cross-linked by type II transglutaminase by peptide map analysis with amino acid sequencing.** The monomer and dimer of recombinant RP S19 were respectively pretreated with TPCK-trypsin, and then subjected to reverse phase HPLC on a C18 column. The solid and broken lines denote the absorbance at 210 nm and acetonitrile concentration, respectively. The horizontal axis denotes the retention time (min). Peptides eluted with a gradient change in the acetonitrile concentration were then subjected to amino acid sequencing analysis. The amino acid sequences of the materials in the peptide peaks attenuated or newly appearing in the map of the dimer compared to that of the monomer are shown on the maps using the single letter code. The amino acid sequences of the materials in the attenuated peaks denoted by asterisks were not analyzed due to their low doses. They are identified only by their retention times.

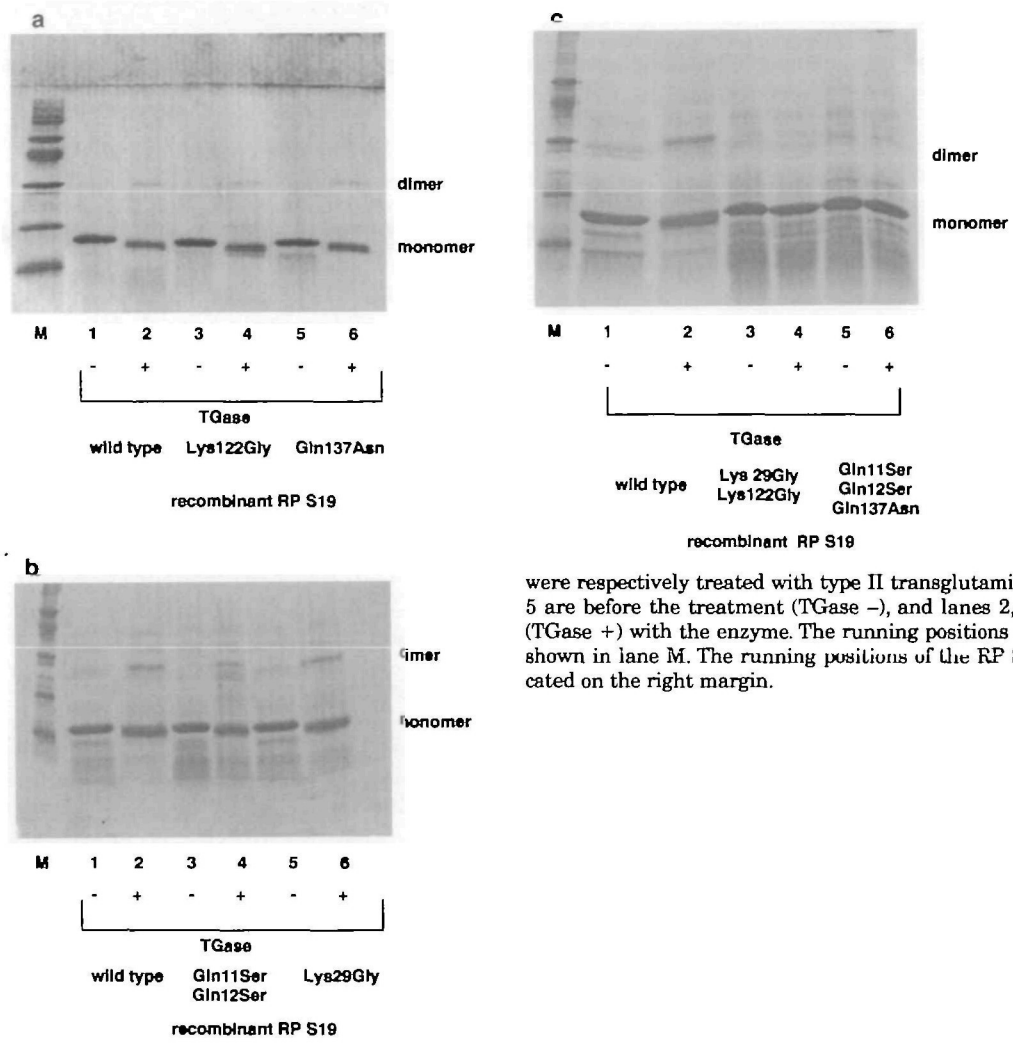

Fig. 11. **Examination of Gln and Lys residues of RP S19 cross-linked by type II transglutaminase using site-directed mutants of RP S19.** The efficacy of the cross-linkage of RP S19 by type II transglutaminase was compared on SDS-PAGE among the wild type, Lys122Gly mutant and Gln137Asn mutant of recombinant RP S19 (a), among the wild type, Gln11Ser-Gln12Ser mutant and Lys29Gly mutant of recombinant RP S19 (b), or among the wild type, Lys29Gly-Lys122Gly mutant and Gln11Ser-Gln12Ser-Gln137Asn mutant (c). The recombinant proteins (0.5 mg/ml) in 10 mM Tris-HCl buffer (pH 7.5) containing 100 mM NaCl, 5 mM $CaCl_2$, and 1 mM DTT were respectively treated with type II transglutaminase (0.5 unit/ml). Lanes 1, 3, and 5 are before the treatment (TGase –), and lanes 2, 4, and 6 are after the treatment (TGase +) with the enzyme. The running positions of the molecular size markers are shown in lane M. The running positions of the RP S19 monomer and dimer are indicated on the right margin.

29Gly and Lys122Gly, the monomer of Gln137Asn-RP S19 possessed chemotactic capacity equivalent to that of its dimer.

DISCUSSION

AsPC-1 cells underwent apoptosis on heat-treatment for 60 min at 43°C. In terms of the TUNEL-positive cell ratio, more than 90% of the cells had undergone apoptosis by 24 h after the heat-treatment (Fig. 1). Since 90% of HL-60 cells underwent apoptosis with the same treatment for 30 min (*3*), AsPC-1 cells seem to be more resistant than HL-60 cells to the apoptosis induction signal.

As in the case of HL-60 cells (*3*), AsPC-1 cells liberate the cross-linked RP S19 dimer during the apoptotic process as the major monocyte chemotactic factor. This is concluded from the results of the present immunologic analyses in which the majority of the monocyte chemotactic capacity in the culture supernatant of AsPC-1 cells was absorbed by immuno-beads bearing either the anti–RP S19 rabbit antibodies or the anti–isopeptide bond monoclonal antibody (Figs. 3 and 4). In terms of the chemotactic capacity, AsPC-1 and HL-60 release similar amounts of the RP S19 dimer during the apoptotic process. From the chronological point of view, the peak of the RP S19 dimer release from apoptotic AsPC-1 cells was at either 24 h or 30 h after the heat-treatment depending upon the culture batch. In the case of HL-60 cells, the apparent peak was at 24 h after the heat-treatment.

The inter-molecular cross-link reaction forming an isopeptide bond(s) is catalyzed by a member(s) of the transglutaminase family. Five major types of mammalian transglutaminases are known. They are factor XIIIa (plasma transglutaminase), keratinocyte transglutaminase (type I), tissue transglutaminase (type II), epithelial transglutaminase (type III), and prostate transglutaminase (type IV). Among them, type II transglutaminase is a ubiquitous enzyme except in the brain and thymus, while factor XIIIa (A′ subunit) is only produced by monocytes/macrophages and megakaryocytes (*17*).

Since the origin of AsPC-1 is a pancreatic ductal cells, the present immuno-cytochemical data demonstrating that AsPC-1 possesses type II transglutaminase but not factor XIIIa are reasonable. An increase in type II transglutaminase with the enzyme activity during the apoptotic process of AsPC-1 was also observed (Figs. 5 and 6). This result is consistent with previous reports on increased type II transglutaminase (*18*) or intracellular transglutainase activity

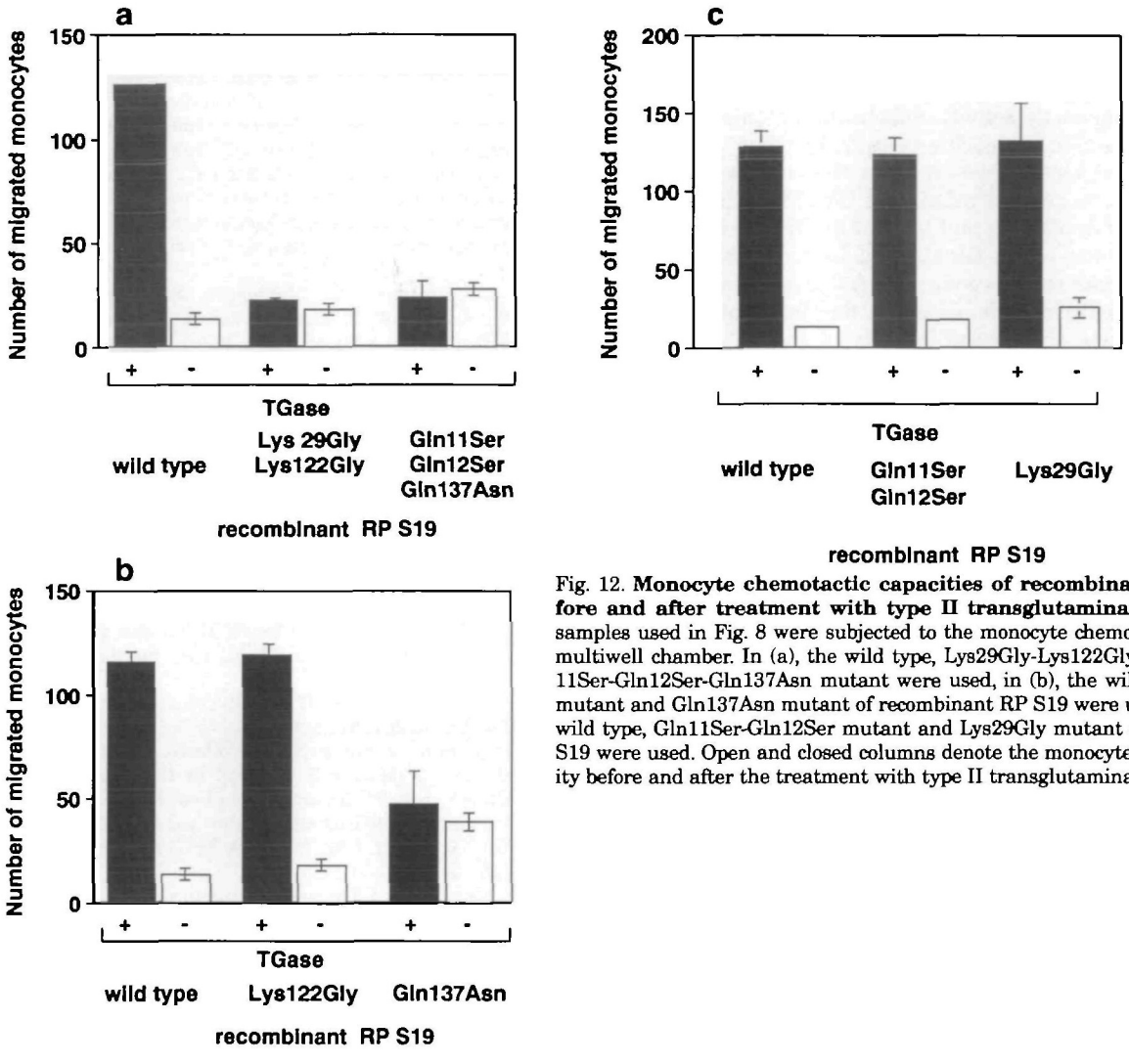

Fig. 12. **Monocyte chemotactic capacities of recombinant molecules before and after treatment with type II transglutaminase.** Aliquots of the samples used in Fig. 8 were subjected to the monocyte chemotaxis assay with a multiwell chamber. In (a), the wild type, Lys29Gly-Lys122Gly mutant and Gln11Ser-Gln12Ser-Gln137Asn mutant were used, in (b), the wild type, Lys122Gly mutant and Gln137Asn mutant of recombinant RP S19 were used, and in (c), the wild type, Gln11Ser-Gln12Ser mutant and Lys29Gly mutant of recombinant RP S19 were used. Open and closed columns denote the monocyte chemotactic activity before and after the treatment with type II transglutaminase, respectively.

(*5*) during the apoptotic process of epithelial cells. The time period of the increased transglutainase activity proceeded that of the monocyte chemotactic RP S19 dimer release by about 6 h in the apoptosis of AsPC-1 cells. When the recombinant RP S19 was incubated with the apoptotic AsPC-1 cell extract containing the enhanced type II transglutainase activity, the monocyte chemotactic activity was generated. Furthermore, the chemotactic activity generation was prevented by pretreatment of the extract with the anti–type II transglutaminase antibodies (Fig. 7). These results strongly suggest that the RP S19 dimer released into the culture supernatant of the apoptotic AsPC-1 cells would have been formed through the type II transglutaminase-catalyzed reaction.

Using the authentic type II transglutaminase, it was here shown that RP S19 is a good substrate of type II transglutaminase as well as of factor XIIIa. Factor XIIIa requires heparin as a cofactor for the cross-linking reaction of RP S19. Since RP S19 and factor XIIIa both bind to heparin, we have assumed that heparin would play a role as a kind of concentrator for RP S19 and factor XIIIa in the enzymatic reaction (*4*). On the other hand, type II transglutaminase did not require heparin for the cross-linking of RP S19; moreover, heparin was found to disturb the enzymatic reaction (Fig. 8). The enzyme molecule would have a second interaction site for RP S19, apart from the catalytic center.

Although the transglutaminases catalyze the isopeptide bond formation between Gln and Lys residues, the consensus amino acid sequences around the substrate Gln and Lys residues preferentially recognized by each transglutaminase are not clear, although there are some tendencies (*19*). The Gln and Lys residues cross-linked by factor XIIIa in the presence of heparin were restricted to Gln137 and Lys122. It is speculated that this restriction would be in part due to steric hindrance by heparin intervening in the multiple site interaction between factor XIIIa and RP S19 molecules. In the case of the cross-linkage of RP S19 by type II transglutaminase, multiple Gln and Lys residues on RP S19 molecules such as Gln12 and Gln137, and Lys29 and Lys122 could be potentially used by this enzyme. This is our interpretation of the results of the trypsin-map analyses (Fig. 10) and site-directed mutagenic analyses (Fig. 11).

However, in terms of the monocyte chemotactic activity, not every type of cross-linked RP S19 dimer can gain it.

The dimer fraction prepared with type II transglutaminase would be a mixture of active and non-active dimers, at least in the *in vitro* reaction system. The use of Gln137 in the dimerization with isopeptide bond formation is essential for gaining of the chemotactic activity. Substitution of one the residues other than Gln137, such as Gln12, Lys29 or Lys-122, seems to have a negligible effect on the chemotactic activity expression, as demonstrated with Gln11Ser-Gln12-Ser-RP S19, Lys29Gly-RP S19 and Lys122Gly-RP S19 (Fig. 12, b and c). In these cases, Gln137 can be cross-linked. These results indicate that a conformational change of the Gln137 residue itself would be essential for the potential but hidden monocyte chemotactic activity of the RP S19 monomer. We assume hydrogen bond(s) formation between the Gln137 residue and an undetermined residue(s) would mask the chemotactic activity of the monomer. This assumption is supported by the partial chemotactic activity of the Gln137Asn-RP S19 monomer (Fig. 12b).

It is known that type II transglutaminase increases during the apoptotic process and plays the major role in the apoptotic body formation (*5*, *19*). Taking this information and our present experimental data together, one can assume that the enzyme which catalyzes the RP S19 dimerization during the apoptotic process would be type II transglutaminase.

Since most nucleated cells possess ribosomes and type II transglutaminase as well as the pro-apoptotic machinery, the dimerization and liberation of RP S19 would be a common event in the apoptotic process. As a consequence of this event, recruitment of monocytes/macrophages and phagocytic clearance of the apoptotic cells occur (*3*). Furthermore, this clearance is associated by the examination of the antigenic information present in the apoptotic cells by the acquired immune system (*20*). As a whole, these processes must be essential and beneficial for a multicellular organism in terms of host defense (*21*).

REFERENCES

1. Nishiura, H., Shibuya, Y., and Yamamoto, T. (1998) S19 ribosomal protein cross-linked dimer causes monocyte-predominant infiltration by means of molecular mimicry to complement C5a. *Lab. Invest.* **78**, 1615–1623
2. Nishiura, H., Shibuya, S., Matsubara, S., Tanase, S., Kambara, T., and Yamamoto, T. (1996) Monocyte chemotactic factor in rheumatoid arthritis synovial tissue. Probably a cross-linked derivative of S19 ribosomal protein. *J. Biol. Chem.* **271**, 878–882
3. Horino, K., Nishiura, H., Ohsako, T., Shibuya, Y., Hiraoka, T., Kitamura, N., and Yamamoto, T. (1998) A monocyte chemotactic factor, S19 ribosomal protein dimer, in phagocytic clearance of apoptotic cells. *Lab. Invest.* **78**, 603–617
4. Nishiura, H., Tanase, S., Shibuya, Y., Nishimura, T., and Yamamoto, T. (1999) Determination of the cross-linked residues in homo-dimerization of S19 ribosomal protein concomitant with exhibition of monocyte chemotactic activity. *Lab. Invest.* **79**, 915–923
5. Knight, C.R.L., Rees, R.C., and Griffin, M. (1994) Apoptosis: a potential role for cytosolic transglutaminase and its importance in tumor progression. *Biochim. Biophys. Acta* **1096**, 312–318
6. Fernandez, H.N., Henson, P.M., Otani, A., and Hugli, T.E. (1978) Chemotactic response to human C3a and C5a anaphylatoxins. I. Evaluation of C3a and C5a leucotaxis *in vitro* under simulated *in vivo* conditions. *J. Immunol.* **120**, 109–115
7. Matsubara, S., Yamamoto, T., Tsuruta, T., Takagi, K., and Kambara, T. (1991) Complement C4-derived monocyte-directed chemotaxis-inhibitory factor. A molecular mechanism to cause polymorphonuclear leukocyte-predominant infiltration in rheumatoid arthritis synovial cavities. *Am. J. Pathol.* **138**, 1279–1291
8. Tsuruta, T., Yamamoto, T., Matsubara, S., Nagasawa, S., Tanase, S., Tanaka, J., Takagi, K., and Kambara, T. (1993) Novel function of C4a anaphylatoxin. Release from monocytes of protein which inhibits monocyte chemotaxis. *Am. J. Pathol.* **142**, 1848–1857
9. Cianciolo, G.J. and Snyderman, R.J. (1981) Monocyte responsiveness to chemotactic stimuli is a property of a sub population of cells that can respond to multiple chemoattractants. *J. Clin. Invest.* **67**, 60–68
10. Falk, W., Goldwin, R.H., and Leonard, E.J. (1980) A 48 well micro chemotaxis assembly for rapid and accurate measurement of leukocyte migration. *J. Immunol. Methods* **33**, 239–247
11. Brendan, C. (1997) Directed mutagenesis using the polymerase chain reaction in Current Protocols in Molecular Biology (Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K., eds.) pp. 8.5.1–8.5.10, John Wiley & Sons, New York
12. Chang, S.K. and Chung, S., II (1986) Cellular transglutaminase. *J. Biol. Chem.* **261**, 8112–8121
13. Lorand, L., Parameswaran, K.N., and Velasco, P.T. (1990) Sorting-out of acceptor-donor relationships in the transglutaminase-catalyzed cross-linking of crystallins by the enzyme-directed labeling of potential sites [published erratum appears in *Proc. Natl. Acad. Sci. USA* 1991; **88**, 2967] *Proc. Natl. Acad. Sci. USA* **88**, 82–83
14. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
15. Gavrieli, Y., Sherman, Y., and Ben-Sasson, S.A. (1992) Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell. Biol.* **119**, 493–501
16. Siebenlist, K.R. and Mosesson, M.W. (1994) Progressive cross-linking of fibrin gamma chains increases resistance to fibrinolysis. *J. Biol. Chem.* **269**, 28414–28419
17. Aeschlimann, D. and Paulsson, M. (1994) Transglutaminases; protein cross-linking enzymes in tissue and body fluids. *Thrombos. Haemostas.* **71**, 402–415
18. Cumings, M. (1996) Apoptosis of epithelial cells in vivo involves tissue transglutaminase upregulation. *J. Pathol.* **179**, 288–293
19. Grootjans, J.J., Groenen, P.J.T.A., and de Jong, W.W. (1995) Substrate requirement for transglutaminases. Influence of the amino acid residue preceding the amine donor lysine in a native protein. *J. Biol. Chem.* **270**, 22855–22858
20. Shrestha, A., Horino, K., Nishiura, H., and Yamamoto, T. (1999) Acquired immune response as a consequence of the macrophage-dependent apoptotic cell clearance and role of the monocyte chemotactic S19 ribosomal protein dimer in this connection. *Lab. Invest.* **79**, 1629–1642
21. Yamamoto, T. (2000) Molecular mechanism of monocyte predominant infiltration in chronic inflammation: Mediation by a novel monocyte chemotactic factor, S19 ribosomal protein dimer. *Pathol. Int.* **50**, 863–871