ORIGINAL CONTRIBUTION

Protease-activated receptor 2 modulates proliferation and invasion of oral squamous cell carcinoma cells☆,☆☆

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Summary Based on our previous finding that protease-activated receptor 2 (PAR-2) regulates hemophagocytosis of oral squamous cell carcinoma (SCC) cells, which induces their heme oxygenase 1–dependent keratinization, we have formulated a hypothesis that PAR-2 functions in wider activities of SCC cells. To confirm this hypothesis, we investigated immunohistochemical profiles of PAR-2 in oral SCC tissues and its functional roles in cell proliferation and invasion in SCC cells in culture. The PAR-2 expression modes were determined in 48 surgical tissue specimens of oral SCC. Using oral SCC–derived cell systems, we determined both gene and protein expression levels of PAR-2. SCC cell proliferation and invasive properties were also examined in conditions in which PAR-2 was activated by the synthetic peptide SLIGRL. PAR-2 was immunolocalized in oral SCC and carcinoma in situ cells, especially in those on the periphery of carcinoma cell foci (100% of cases), but not in normal oral epithelia. Its expression at both gene and protein levels was confirmed in 3 oral SCC cell lines including ZK-1. Activation of PAR-2 induced ZK-1 cell proliferation in a dose-dependent manner. PAR-2–activated ZK-1 cells invaded faster than nonactivated ones. The expression of PAR-2 is specific to oral malignancies, and PAR-2 regulates the growth and invasion of oral SCC cells.

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1. Introduction

Early detection of early stage oral cancer, especially squamous cell carcinoma (SCC), often translates to a satisfactory clinical outcome [1]. To this end, we have developed several aids for the evidence-based histopathologic diagnosis of oral carcinoma in situ (CIS), which is the most critical disease entity among oral borderline malignancies. The precise diagnosis of CIS at surgical margins
might determine prognoses of individual patients because according to our recent studies, CISs would recur as CISs or SCCs in 39.2 months [2] or in 12.3 months [3], when CISs are left behind at surgery.

To avoid subjectivities in the differential diagnosis of oral CIS, we have introduced combined immunohistochemistry for keratin (K) 19, K13, and Ki-67 for diagnostic criteria of CIS with the conceptual background that the second basal cell layer is the proliferating center of the oral mucosal epithelia and that the basal cell differentiation is determined with K19 expression and the prickle cell differentiation is recognizable with K13 expression [4]. We have also demonstrated the genetic mechanism for the loss of K13 as well as the reciprocal loss of K13 and emergence of K16/K17 in oral CIS [5,6]. To consolidate our diagnostic criteria of oral CIS, we have introduced some other immunohistochemistry for podoplanin [7,8], perlecan [9-12], matrix metalloproteinase (MMP) 7 [10], tenasin [12], and β-catenin/E-cadherin [11] as diagnostic aids. More recently, we have also emphasized the formation of intraepithelial blood vessels as one of the histopathologic characteristics of oral CIS and as a driving force for dyskeratosis among cancer cells [13].

Seeking the molecular mechanism of abnormal keratinization in oral CIS and SCC, we have come to find that the expression levels of K17 and K10 were elevated in round-shaped dyskeratosis in CIS as well as in keratin pearls in SCC by hemoglobin derived from extravasated erythrocytes via intraepithelial blood vessels [13,14]. Interestingly, this hemophagocytosis activity is mediated by protease-activated receptor 2 (PAR-2) through heme oxygenase 1 activation pathways [14], whereas PAR-2 had previously been known to up-regulate keratinocyte phagocytosis [15]. PAR-2 is one of the four PAR family members. The PAR family members are G-protein–coupled receptors that regulate cellular motility, growth and differentiation, and gene transcription [16]. PARs are uniquely activated by cleavage of their NH2-terminal domains by serine proteinases for exposing new NH2-termini, which further function as tethered ligand domains for their activation sites [17].

Although PAR-2 is the least understood among the four family members, it has been reported to be expressed in various cell types, including keratinocytes or epithelial cells of the kidney and intestines, vascular endothelial cells, and neural and muscle cells [18]. Trypsin has been considered as its main activator, whereas in vitro, the synthetic peptide SLIGKV, which mimics the NH2-terminal portion of the human receptor, and SLIGRL, a murine homolog, are known to activate PAR-2 [19]. Functionally, PAR-2 activation is implicated in a broad spectrum of pathophysiologic processes, including regulation of vascular [20] and inflammatory [21] responses through cell membrane trafficking. In addition, recent reports have emphasized that PAR-2 is involved in cancer cell behaviors in the breast [22], stomach [23], prostate [24], pancreas [25], colon [26], and cervix uteri [27]. However, in terms of its detailed roles, more extensive studies are needed to elucidate whether PAR-2 could be a neoplastic phenotype.

As mentioned above, it remains totally unknown how PAR-2 functions in oral SCC or CIS in terms of their growth or invasion, other than its hemophagocytosis-related expression, which we have determined in oral SCC cells [14]. The aims of this study are to characterize PAR-2 expression profiles in the oral mucosal epithelia from normal, dysplastic, CIS, and SCC stages and to determine the effect of PAR-2 activation by agonist peptides on oral SCC cells in their proliferation and invasion potentials.

2. Materials and methods

2.1. Tissue samples

A total of 48 surgical specimens of oral SCC, in which areas of normal epithelia and foci of epithelial dysplasia and CIS were simultaneously contained, were randomly selected from surgical pathology files of the Division of Oral Pathology, Department of Tissue Regeneration and Reconstruction, Niigata University Graduate School of Medical and Dental Sciences, during the 15-year period from 1997 to 2010. The surgical specimens were fixed in 10% formalin and routinely embedded in paraffin. One set of 5-μm serial sections was stained with hematoxylin and eosin and used to reevaluate the pathologic diagnosis. The other sets were used for immunohistochemical investigations with the antibodies described below. The experimental protocol for analyzing surgical material was reviewed and approved by the Ethical Board of the Niigata University Graduate School of Medical and Dental Sciences (Oral Life Science).

2.2. Antibodies

A monoclonal mouse antibody against human PAR-2 (clone SAM11, IgG2a) was purchased from Zymed Laboratories (South San Francisco, CA). Rabbit polyclonal antibodies against mouse immunoglobulin G (IgG) was obtained from Dako (Glostrup, Denmark). Antibodies against K10, K13, K17, K19, Ki-67, and perlecan were used for objective categorization of dysplasia, CIS, and SCC, as described elsewhere [5-8].

2.3. Enzyme immunohistochemistry

Deparaffinized sections were autoclaved in citrate buffer (pH 6.0) at 121°C for 10 minutes to restore antigenicity. After antigen retrieval treatments, the sections were rinsed in 0.01 M phosphate-buffered saline (PBS) and treated in 0.3% H2O2 in methanol at room temperature for 30 minutes for blocking endogenous peroxidase activities. The sections were then incubated in 5% skimmed milk in PBS containing 0.05% Triton X-100 (PBST) at 37°C for 30 minutes to block
nonspecific protein bindings, and then the sections were incubated with the primary antibodies at 4°C overnight. After rinsing in PBST, the sections were incubated with goat antibodies against rabbit or mouse IgGs conjugated with peroxidase-labeled dextran polymers (ChemMate EnVision; Dako) at room temperature for 1 hour. After rinsing with PBS, peroxidase reaction products were visualized by incubation with 0.02% 3,3′-diaminobenzidine (Dojindo Laboratories, Kumamoto, Japan) in 0.05 M Tris-HCl solution (pH 7.4) containing 0.005% H2O2. The sections were then counterstained with hematoxylin and mounted. For control studies on the antibodies, the primary antibodies were replaced with preimmune rabbit or mouse IgGs [5–8].

2.4. Reagents

A PAR-2–specific activating peptide, SLIGRL-NH2, and its inactive control, ISLLRG-NH2, were synthesized by Japan BioService (Asaka, Japan). Their peptide purity was greater than 95% as determined by high-performance liquid chromatography.

2.5. Cells

ZK-1 cells, a human carcinoma cell system, have been established from a well-differentiated SCC arising in the tongue of a 53-year-old man [12], whereas MK-1 cells, another SCC cell system with highly metastatic potential, have been established from a submandibular lymph node metastatic focus of a tongue SCC from a 57-year-old man [28]. ZK-1 and MK-1 cells were seeded in 25-cm² plastic flasks at a cell concentration of 4 × 10⁴ in 5 mL Dulbecco’s minimal essential medium (DMEM; Gibco, Thermo Fisher Scientific, Waltham, MA) containing 10% fetal calf serum (Gibco), 50 IU/mL penicillin, and 50 µg/mL streptomycin (Gibco) and cultured at 37°C in humidified 5% carbon dioxide 95% air atmosphere. When they reached 80% confluency, they were subjected to immunofluorescence, Western blotting, and cell proliferation and invasion assays, as described later.

2.6. Immunofluorescence

ZK-1 and MK-1 cells were plated at a cell concentration of 1.2 × 10⁴ cells/well and cultivated for 7 days in chamber slides (Nunc Lab-Tek II Chamber Slide System; Thermo Fisher Scientific). Every 24 hours after plating, 1 slide each was washed with PBS and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) on ice for 30 minutes and then permeabilized by adding 0.2% Triton X-100 to the fixative on ice for 20 minutes. After overnight treatment with 5% skim milk in PBST to prevent nonspecific protein binding, the cells were incubated with the anti–PAR-2 antibody for 1 hour at room temperature, washed with PBST, and then incubated with secondary antibodies (Alexa Fluor 568-conjugated goat anti-mouse IgG; Invitrogen, Thermo Fisher Scientific) diluted at 1:200 in PBS for 30 minutes each at room temperature. Finally, cells and tissues were counterstained with Cellstain Hoechst-33258 solution (Dojindo) diluted at 1:100 in PBS. For control studies, the primary antibodies were replaced with preimmune mouse or rabbit IgGs. After incubations with the antibodies, plastic frames were removed from slide glasses, to which cover glasses were mounted with 90% glycerol in PBS. The slides were examined by a Nikon Eclipse E600W immunofluorescence microscope (Nikon, Tokyo, Japan) equipped with an ORCA-ER digital camera (Hamamatsu Photonics, Hamamatsu, Japan).

2.7. Western blotting

ZK-1 and MK-1 cell cultures in 60-mm dishes at day 5 were lysed with 350 µL of lysis buffer comprising 50 mM HEPES, pH 7.4; 150 mM NaCl; 1% Triton X-100; a protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan); 1 mM PMSF; 1 mM Na3VO4; and 10 mM NaF, and supernatants of the cell lysates were recovered. After the total protein was determined, precleared lysates were incubated with the antibody overnight, and immune complexes were isolated with protein G-Sepharose (GE Healthcare UK, Little Chalfont, UK). Immunoisolated materials were dissolved in Laemmli’s sample buffer, boiled for 5 minutes, and centrifuged at 10 000 × g for 5 minutes to remove beads. The supernatants were applied for sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the gels were transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Inc., Hercules, CA). After incubation with 0.5% enhanced chemiluminescence blocking agent (GE) in 50 mM Tris-buffered saline (pH 7.4) containing 0.1% Tween-20 (TTBS) for 1 hour at room temperature, the membranes were further incubated overnight at 4°C with the anti–PAR-2 antibody or preimmune IgG diluted with TTBS (1:100). After washing with TTBS, the membranes were reacted with secondary antibodies (ChemMate Envision; Dako, diluted at 1:1000 in TTBS) for 1 hour at room temperature. Target protein bands were visualized by ECL Plus Western blotting detection reagents (GE), according to the manufacturer’s instructions [8].

2.8. RNA isolation and semiquantitative reverse-transcription polymerase chain reaction

ZK-1 and MK-1 cells cultured in 60-mm dishes were lysed with 1 mL ISOGEN (Nippon Gene, Tokyo, Japan), and total RNA was then extracted from the cell lysate according to the manufacturer’s instructions. After RNA yields were determined spectrophotometrically, complementary DNA was synthesized from 5 µg of each RNA sample with the Invitrogen SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific) followed by polymerase chain reaction (PCR) using 1 µL aliquot of complementary DNA samples, which was carried out in an Astec thermal cycler PC-800 (Astec, Fukuoka, Japan) with PAR-2 as a target gene. The primer sets for PAR-2 were...
5'-GCCAT CTCGC TAGCA GCCTC TC-3' (forward) and 5'-GATGA CAGAG AGGAG GTACG CC-3' (reverse), and their thermocycling protocol during 30 amplification cycles was as follows: denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, extension at 72°C for 1 minute, and extension at 72°C for 7 minutes. To ensure semiquantitative results of the reverse-transcription polymerase chain reaction (RT-PCR) assays, the number of PCR cycles for each set of primers was checked to determine whether it was in the linear range of amplification. Each 10 µL of the amplified DNA fragments was analyzed by electrophoresis on 3% agarose gel (Lonza NuSieve 3:1; Thermo Fisher Scientific), and bands for the PCR products were visualized on the gels by ethidium bromide staining under ultraviolet light [8,14].

2.9. Cell proliferation assay

ZK1 cells lines (4 × 10⁴ cells/flask) were cultured as mentioned above for 48 hours. The media were replaced with serum-free starvation media containing 0.1% fetal calf serum and incubated for 48 hours to maintain them quiescent. The cells were then seeded in 96-well microplates (5000 cells/well) and incubated for 48 hours in serum-free culture media containing PAR-2 agonist peptide SLIGRL at concentrations of 50, 100, and 200 µM or in those containing control peptide ISLLRG (50, 100, and 200 µM). At the end of incubation, cell numbers were determined by CellTiter 96 AQueous One Solution Cell Proliferation Assay Kit (Promega, Madison, WI). Experiments were done in triplicate and repeated 3 times [8].

2.10. Matrigel invasion assay

Onto inserts of BD BioCoat Matrigel Invasion Chamber (BD Falcon, 12-well plates, pore size 8 µm; BD Biosciences, Bedford, MA), equal numbers (1 × 10⁴) of ZK-1 cells were seeded in serum-free DMEM. Then, 200 µM PAR-2 agonist peptide SLIGRL or 200 µM control peptide ISLLRG was added to the upper compartment of the chamber and kept at 37°C for 24 hours. As a chemoattractant, the lower compartment contained DMEM supplemented with 10% fetal calf serum. At the end of the incubation periods, cells from the upper surface of the filters were wiped off with cotton swabs. The lower surface of the filters was stained with DiffQuick (Baxter AG, Volketswil, Switzerland). The number of cells having invaded to the bottom of the chamber was counted in 10 randomly selected unit fields (0.2 × 0.2 mm = 0.04 mm²) on the light microscope equipped with an Olympus OCM eye piece micrometer (Olympus, Tokyo, Japan) at magnification ×400. The mean number of cells was calculated per field. Three sets of experiments were carried out, each in triplicate [29].

2.11. Quantitative and statistical analysis

Because all the experiments were done in triplicate, values from the 3 experiments were averaged and plotted with SD values. They were analyzed by one-way analysis of variance using GraphPad Prism software, version 5.0 (GraphPad Software, San Diego, CA). Differences were considered statistically significant at P < .05.

3. Results

3.1. Immunohistochemistry

Oral epithelial lesions in the surgical specimens were categorized by combined immunohistochemistry for K19, K13, K17, Ki-67, and perlecain, as described elsewhere [5-8]. In normal epithelia of the oral mucosa (Fig. 1A), PAR-2 was not demonstrated, whereas it was strongly localized in skeletal muscle cells (Fig. 1B). PAR-2 was not definitely positive in hyperplastic or mild dysplastic conditions (not shown). However, in moderate epithelial dysplasia with two-phase appearances [4,6-11] (Fig. 1C), it was definitely but not uniformly positive in the lower half of the epithelial layer (Fig. 1D). Its diffuse staining was demonstrated occasionally in patches within the cytoplasm or along the cell border. In CIS with irregular-shaped rete ridges (Fig. 1E), PAR-2 was more intensively and more widely positive in carcinoma cells than in dysplastic cells (Fig. 1F). In rete ridges with narrow projection, which were suggestive of microinvasion (Fig. 1E), PAR-2 was localized in basal/peripheral cells of CIS foci (Fig. 1F). In SCC (Fig. 1G), fine granular signals for PAR-2 were enhanced and limited in peripheral cells of each carcinoma cell nest, whereas keratinized cells contained no apparent signals (Fig. 1H). PAR-2 expression was confirmed in all (100%) examined cases of CIS and SCC. The results indicated that PAR-2 expression was dependent on the malignant grade and that PAR-2+ cell areas were overlapped with those with cell proliferative activity or those with invasive tendencies.

3.2. Immunofluorescence

PAR-2 started to be expressed in ZK-1 cells at 24 hours after plating (Fig. 2A). Its fine to coarse granular immunofluorescence signals were scattered over the cytoplasm. On and after day 3, when ZK-1 cells formed colonies, PAR-2 was localized in the periphery of the cytoplasm, indicating that PAR-2 was trafficked to the cell membrane of proliferating and spreading cells (Fig. 2B). MK-1 cells, which were more densely packed than ZK-1 cells, showed similar immunofluorescence profiles, although their signals were more finely granular than those of ZK-1 cells (Fig. 2C and D). Those expression patterns were basically the same as those obtained in oral CIS and SCC tissue sections.

3.3. RT-PCR and Western blotting

In Western blotting experiments after immunoprecipitation of cell lysates with the anti–PAR-2, doublet bands at 46 to 48 kDa were obtained in both ZK-1 and MK-1
cells (Fig. 3A). Although the two cell types showed exactly the same doublets, their signal intensities in MK-1 were always stronger than those in ZK-1 cells. RT-PCR revealed a single band with 342 bp in ZK-1 as well as MK-1 cells, and PAR-2 relative expression levels were higher in MK-1 than those in ZK-1 (Fig. 3B). The higher PAR-2 protein levels in MK-1 cells were consistent with the RT-PCR data.
3.4. Cell proliferation assay

To evaluate roles of PAR-2 in SCC cell proliferation, ZK-1 cells were cultured for 48 hours in the presence of activation peptide (SLIGRL) or control peptide (ISLLRG) at concentrations at 50, 100, and 200 μM each in serum-free culture media. As shown in Fig. 4, ZK-1 cells increased in number in a dose-dependent manner. The differences in effect between agonist and control peptides were significant at 100 and 200 μM (P < .001).

3.5. Cell invasion assay

To examine whether the agonist peptide has an effect on the invasive activity of ZK-1 cells, Matrigel invasion assays were performed. ZK-1 cells were cultured for 24 hours in the presence or absence of 200 μM activation peptides or in the presence of 200 μM control peptides. As shown in Fig. 5, ZK-1 cells invading the lower surface of culture insert membranes increased in number when the activation peptide was added (Fig. 5A). There were no apparent differences between those without peptide (Fig. 5b) or with control peptide (Fig. 5C). The differences between the agonist peptide and controls, however, were statistically significant (P < .001) (Fig. 5D). The results indicated that the PAR-2–activated ZK-1 cells were more invasive than those in which PAR-2 was not activated.

4. Discussion

The present study clearly demonstrated that PAR-2 expression levels were enhanced in neoplastic conditions of the oral mucosal epithelia. The oral CIS/SCC–specific PAR-2 expression was contrasted with the no PAR-2 expression in normal or hyperplastic epithelia. Since there were some expressions in dysplastic epithelia, PAR-2 seemed to be expressed dependently on the malignant degrees of oral squamous epithelial cells. Thus, PAR-2 is now considered as one of the useful immunohistochemical markers for diagnosing oral malignancies. In our tissue-level investigation, PAR-2 was especially pronounced in SCC cells in their proliferating zone or in the invasive end of CIS. In addition, our functional study showed that PAR-2 activation induced SCC cell proliferation in a dose-dependent manner and that PAR-2–activated cells invaded faster than nonactivated ones. These findings indicate that PAR-2 is needed for proliferation as well as invasion of oral SCC cells.

The PAR-2 expression profiles have not been well elucidated in the oral mucosal epithelium, although the obscure expression of PAR-2 has been documented in normal epidermis [18]. Thus, the present study provides the first evidence of PAR-2 expression in the oral mucosa, in which its expression was dependent on malignant degrees of epithelia. In the uterine cervix, PAR-2 has been...
immunolocalized in SCC cells at both tissue and cellular levels [27]. Thus, PAR-2 expression seems to be very specific to carcinoma cells in squamous epithelium-covering mucosae.

We have paid our attention to PAR-2 because, in our previous study on hemophagocytosis of oral SCC cells, we reported that SCC cells were differentiated (keratinized) by hemoglobin-mediated PAR-2[14]. However, PAR-2 expression is mediated not only by hemophagocytosis but also by other activating pathways, such as trypsin-like serine proteinases [17]. As the enhanced PAR-2 expression profiles have been correlated to metastatic potentials of cervical cancer [30] as well as to poor prognoses in breast [22] and ovarian [31] cancers, we expected that PAR-2 should be activated by various factors in the invading front of oral SCC.

The activation of PAR-2 by trypsin or the other PAR-2 agonists has been demonstrated in cancer cells from the breast [22], stomach [23], prostate [24], pancreas [25], colon [26], and cervix uteri [27]. Several kinds of cancer cells including human colon, stomach, lung, and breast carcinomas were able to produce trypsin, trypsinogen, trypsin-line proteins, and trypsin inhibitors [32]. In patients with Borrmann type IV gastric cancer (limitis plastica), serum trypsin released from carcinoma cells has been detected at nanomolar concentrations [33]. In addition, blood vascular endothelial cells in the vicinity of gastric cancer cell nests also expressed trypsin, whereas those distant from cancer cells did not [34], although D’Andrea et al [18] reported that PAR-2 was expressed even in normal blood vascular endothelial cells. PAR-2 may facilitate cancer cell proliferation and invasion in trypsin-rich extracellular milieux, where PAR-2 on the cancer cell surface may be activated with trypsin derived from both cancer cells themselves and vascular endothelial cells. Because the expression of trypsin in oral SCC has not been well investigated, it is necessary in the next step to determine its expression profiles at tissue and cellular levels.

The molecular mechanism by which PAR-2 promotes cancer cell proliferation and invasion remains poorly understood. However, PAR-2 has been shown to transactivate epidermal growth factor receptor (EGFR) in colon cancer cells for their proliferation [26], which must be further supported by many lines of evidence that EGFR is highly expressed in various cancer cell types including oral SCC [35]. Furthermore, the fact that promotion of metastasis by PAR-2 signaling has been explained by its effect on rearrangement of actin filaments for pseudopodia formation, which eventually results in RhoA-dependent cell motility through the Rac/p21-activated kinase pathway [15,36].
Moreover, PAR-2 has been shown to be capable of regulating the angiogenic process mediated by angiogenic factors [37].

In conclusion, based on the present in vivo and in vitro results and lines of evidence documented in the literature, it is possible to conclude that PAR-2 is required for growth and invasion of oral SCC cells and that it is a novel immunohistochemical marker for histopathologic diagnosis of oral malignancies, in addition to those we have already established [4-13,28], especially when the differential diagnosis of CIS from epithelial dysplasia is difficult. In the next step, the molecular mechanism for PAR-2 activation in the invading front of oral SCC should be investigated more in detail to open a research field toward an anticancer therapy in which repression of PAR-2 should be targeted.

References


Fig. 5 PAR-2 effect on oral SCC cell invasion. Transwell invasion assays of ZK-1 cells with (A, B) or without (C) PAR-2 activation by activation peptide SLIGRL (A) or control peptide ISLLRG (B) (200 μM) for 24 hours. D, Invading cell counts by DiffQuick stain from triplicate experiments. Solid bar, activation peptide; striped bar, control peptide; open bar, untreated. ZK-1 cells that migrated onto the bottom surfaces of inserts were counted in unit squares. In the presence of activation peptide SLIGRL (A), ZK-1 cells invaded more quickly than in the other two conditions with control peptide ISLLRG (B) or without peptides (C). As seen in a bar graph for cell count (D), ZK cell invasion abilities were significantly enhanced only by the activation peptide. *P < .001.


