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Original Article

Reduced expression of nuclear factor kB in oral mucosa undergoing preoperative chemoradiotherapy

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ABSTRACT

Radiotherapy as well as chemotherapy in head and neck cancer induces severe oral mucositis. Even after healing of the mucositis, however, the oral mucosa looking atrophic is known to be susceptible to injury and infection. In order to investigate such vulnerability of mucosa, we immunohistochemically studied the expressions of Ki-67, proliferating cell nuclear antigen (PCNA), cyclin D_1, nuclear factor (NF)-kB, and keratinocyte growth factor (KGF) receptor in the oral mucosal keratinocytes undergoing preoperative concurrent chemoradiotherapy for oral cancer, compared with those of the oral mucosa without such therapy. As a result, the expressions of Ki-67, PCNA, and cyclin D_1 were decreased in the chemoradiotherapy-treated oral keratinocytes. Interestingly, NF-kB expression, which is known to be enhanced in oral mucositis, was reduced after chemoradiotherapy. The chemoradiotherapy had no effect on the expression of KGF receptor in oral keratinocytes. In conclusion, the vulnerability of oral mucosa undergoing chemoradiation may be associated with reduced NF-kB expression and impaired growth activity.

1. Introduction

Radiotherapy, as well as chemotherapy, on head and neck cancer induces severe oral mucositis [1]. This radiation-induced or anticancer drug-induced oral mucositis spontaneously heals 3–4 weeks after treatment. In particular, the oral mucosa once exposed to radiation appears to become atrophic and susceptible to injury even after the complete reepithelialization of mucosa. We often experience that the mechanical stress such as an irritation by food and improper denture on such radiated mucosa easily induces ulceration even if the oral mucosa appears to be healthy. If such ulceration occurs on gingiva, it may cause infection of alveolar bones and trigger the occurrence of osteoradionecrosis of the jaw. Hence, oral care for patients with head and neck cancer after radiotherapy or chemoradiotherapy is crucial. However, the reason why the oral mucosa exposed to radiation is atrophic and vulnerable remains unknown.

In order to understand the biological properties of oral mucosa undergoing radiotherapy or chemotherapy, the accumulating knowledge about the mechanisms of radiation- or chemotherapy-induced oral mucositis may be helpful.

Oral mucositis is an adverse effect arising in almost all patients with head and neck cancer undergoing radiotherapy or chemotherapy, which impairs the food intake because of severe oral pain and bleeding, and worsens the quality of life of the patients [2]. It is a great benefit to the patients if radiation- or anticancer drug-induced oral mucositis is well relieved [3,4]. The management for oral mucositis should be based on the pathobiology for radiation- or anticancer drug-induced oral mucositis.

In radiotherapy, the onset of oral mucositis is a biologically complex process [5]. It is thought that radiation damages two types of cells in oral mucosa, squamous epithelial cells (keratinocytes) and submucosal stromal cells (fibroblasts and vascular endothelial cells). Radiation and radiation-generated reactive oxygen species (ROS) damage DNA itself, inhibit cell proliferation, and trigger the apoptosis or necrosis of oral keratinocytes, which gives rise to the breakdown of squamous cell layer to result in ulceration [5]. In addition, the arrest of cell cycle suppresses the mucosal regeneration and the wound healing process. As a result, refractory oral mucositis develops.

In the initiation stage of radiation-induced mucositis, DNA damage and ROS trigger the activation of transcription factors such as nuclear factor-kB (NF-kB), AP-1, and p53 [5–7]. Among them, NF-kB seems to be a key transcription factor in the establishment of mucositis [8]. NF-kB activation can upregulate the expression of

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Table 1
Patients characteristics.

	Group S ($n = 10$)[a]	Group Ra ($n = 10$)
Male:female	3:7	8:2
Age (years)	63.5 (50–82)	57.2 (49–72)
Tumor location		
Tongue	4	1
Lower gum	3	3
Oral floor	2	4
Buccal mucosa		1
Upper gum	0	1
Histopathological response[b]		
Grade IIa	–	2
Grade IIb	–	2
Grade III	–	1
Grade IV	–	5

[a] Group S, the cases without radiotherapy; Group Ra, the cases with chemoradiotherapy.
[b] Histopathological response assessment. Grade IIa, obliteration of tumor structures was mild and viable tumor cells were observed in more than 25%; Grade IIb, obliteration of tumor structures was severe and viable tumor cells were observed in lessthan 25%; Grade III, only non-viable tumor cells present; Grade IV, tumor cells were not observed.

pro-inflammatory cytokines including tumor necrosis factor (TNF) α, interleukin (IL)-1β, and IL-6 [8]. It seems likely that the increased levels of these cytokines induce inflammatory reactions in oral mucosa, promote the damage of the underlying connective tissues, reduce epithelial oxygenation, and ultimately result in epithelial basal cell death and injury. Since TNFα and IL-1β are efficient activators of NF-kB, the repeated NF-kB activation by them may amplify the mucosal damage in a vicious circle [5].

If NF-kB plays a central role in the development of radiation-induced mucositis, it may be also implicated in the vulnerability of normal oral mucosa undergoing chemoradiotherapy. However, there have been few reports studying the expression of NF-kB in human oral mucosa after chemoradiotherapy. Usually we have no occasions to take specimens from normal oral mucosa after the healing of oral mucositis. In the present study, instead, we observed the expression of NF-kB in the non-cancerous oral mucosa which was present in the specimens dissected from oral cancer patients undergoing preoperative concurrent chemoradiotherapy, comparing with those from the patients without chemoradiotherapy.

2. Materials and methods

2.1. Subjects

Twenty consecutive patients with primary oral squamous cell carcinoma (OSCC), who presented to the Department of Oral and Maxillofacial Surgery, Graduate School of Medical Sciences, Kumamoto University Hospital from January 2003 to July 2003, followed with tumor surgery, were divided into two groups. One group ($n = 10$) consists of the patients who underwent preoperative concurrent chemoradiotherapy, that is radiotherapy (total 30 Gy; 2 Gy per a day for 15 days) combined with oral administration of S-1 for 14 days, prior to surgery (Group Ra), and the other ($n = 10$) is the group of the patients who were operated on without preoperative chemoradiotherapy (Group S). The clinical details of both groups are shown in Table 1. In all the patients of Group Ra, oral mucositis occurred, which was treated with lidocaine-containing oral rinse and corticosteroid powder. Three to four weeks after the end of chemoradiotherapy, when oral mucositis was almost reepithelialized and healed, tumor surgery was performed.

2.2. Immunohistochemical staining

Four-micrometer-thick histological sections of 4% paraformaldehyde fixed, paraffin embedded tissues were formed from the tumor specimens, mounted, and air-dried. The sections were deparaffinized in xylene and rehydrated in ethanol, and endogenous peroxidase was blocked by immersion in methanol containing 0.3% hydrogen peroxidase for 30 min. The sections were incubated overnight at 4 °C with their respective primary antibodies, then for 30 min at room temperature with the peroxidase-conjugated anti-mouse or anti-rabbit IgG Ab, followed by staining with diaminobenzidine (Histofine MAX-PO kit, Nichirei, Tokyo, Japan) [9]. The primary antibodies used were as follows: mouse anti-cyclin D_1 monoclonal antibody (Invitrogen, Life Technologies Japan Ltd., Osaka, Japan), mouse anti-Ki67 monoclonal antibody (Invitrogen, Life Technologies Japan Ltd.), rabbit anti-keratinocyte growth factor (KGF) receptor polyclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), rabbit anti-NF-κB p65 polyclonal antibodies (Santa Cruz Biotechnology, Inc.), mouse anti-PCNA monoclonal antibody (Santa Cruz Biotechnology, Inc.).

Two independent researchers (K.Y. and Y.T.) determined the microscopic views on the areas located away from tumor nests, where only the normal keratinocytes were observed without any cancerous cells and atypical cells and which appeared to be best stained, and they counted the number of all the cells and the positively stained cells in the squamous cell layer, respectively. The rates of the positively stained cells were calculated as the positive cell numbers per total cell numbers. The average rates from two researchers were regarded as the results. Statistical significance of the rates of the positively stained cells between Group S and Group Ra was assessed with paired one-way ANOVA, where appropriate p-value of <0.05 was considered to be significant.

3. Results

We took the non-cancerous oral mucosa specimens from the extirpated oral cancer tissues, which were located away from tumor nests and consisted of normal keratinocytes. Oral mucosa in Group Ra had been exposed to total 30 Gy of radiation concurrently coupled with S-1 administration 3–4 weeks before, while the tumors in Group S were treated surgically without chemoradiotherapy. The difference between both groups is whether the given oral mucosa underwent chemoradiotherapy or not.

In order to study the effects of chemoradiotherapy on oral mucosa, at first, we immunohistochemically investigated the expression of several cell growth markers such as Ki-67, PCNA, and cyclin D_1 in the oral keratinocytes from both groups (Fig. 1). The rates of the positive cells expressing Ki-67 were 6.0% in Group S and 1.1% in Group Ra. For the expression of PCNA, the rates of the positive cells in Group S and Group Ra were 26.8% and 9.4%, respectively. Like Ki67 and PCNA, 8.9% of the keratinocytes in Group S and 2.7% of those in Group Ra expressed cyclin D_1 (Fig. 1). Statistically, there was a significant difference in the expression of each growth marker between both groups ($p < 0.05$). The growth activity of irradiated keratinocytes in oral mucosa seemed to be reduced after the mucositis previously arising in the same area was healed.

Sonis has reported that a transcription factor NF-kB plays a critical role in the occurrence of radiation-induced oral mucositis [5]. NF-kB activates not only the expression of pro-inflammatory cytokines, leading to mucositis, but also the expression of cyclin D1. In this context, the expression of NF-kB was studied immunohistochemically using anti-p65 subunit of NF-kB antibodies (Fig. 2). The keratinocytes in Group S contained 8.5% of the NF-kB-positive cells, which were mainly located in basal cell layer, while Group Ra