Biological Behavior of basaloid cells in Oral Carcinoma In-situ

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Abstract
Carcinoma in-situ (CIS) is defined as a true and not-yet invasive neoplasm. Histopathologically, it shows basaloid cell formation known as dysplastic cells mixed with normal differentiated cells in the epithelium. Microscopically, it is difficult to distinguish normal basal cells and carcinomatous cells, also how their biological behavior and how they reach the superficial layer are still unknown. To recognize basaloid cells, their biological behavior in order to carry-out a definitive histopathological diagnose and to know how their possibility replace whole layer of epithelium. Twenty cases of formalin-fixed paraffin sections of hyperplasia, 20 cases mild and 15 cases moderate squamous epithelial dysplasia (SED) as a control, and 15 cases of CIS were subject to Ki-67 immunostaining for proliferating cells and Tunel staining for apoptotic cells. Hyperplasia and mild SED cases expressed Ki-67 located in the parabasal layer, while Ki-67 was extended distributed in basal, parabasal up to superficial layer of moderate SED and CIS. Hyperplasia and mild SED cases showed apoptotic cells located in keratin layer, in contrast moderate SED showed apoptotic cells in prickle layer while CIS, apoptotic cells located in keratin layer and basal layer. The basaloid cells as dysplastic or carcinomatous cells occurred in parabasal layer then expanding to two directions; basal layer and prickle layer. It suggested that apoptotic cells which occurred in prickle layer made basaloid cells able to reach until superficial layer. Therefore, the basaloid cells of CIS replaced whole layer of epithelium but their biological behavior was still definite toward keratinization.

Keywords: Oral, CIS, dysplastic, Carcinoma.

INTRODUCTION
Recently, the incidence of oral cancer increased in many countries such as Japan, India, and other Asian countries. In Japan, the mortality of oral carcinoma has increased seven times in last 50 years (The Japanese Society for Oral Pathology, 2005). The etiology of oral carcinoma has been known as multifactor such as smoking, alcohol consumption and habits. In America and Europe, alcoholic addiction and smoking are main factors for increased oral cancer incidences (Gould, 1995). Ash dan Ward, (1992) reported that genetics also has a role play in cancerization (Ash et. al., 1992).

The concept of oral CIS is the most important aspect in the present criteria, because Squamous Epithelial Dysplasia (SED) and invasive Squamous Cell Carcinoma (SCC) can be easily discriminated, however, when and what stage could be called as CIS is still unclear and controversial because there are no fixed criteria yet.

Histopathologically, epithelial dysplasia has been conventionally classified into three grades: mild, moderate, and severe (Carcinoma In-situ), although the relationship of these grades to the subsequent development of cancer has not been fully clarified. The WHO Histological Typing of Cancer and Precancer of the Oral Mucosa, 2nd
Biological Behavior of basaloid cells in Oral Carcinoma In-situ edition (1997) proposed thirteen characteristic histological changes that may occur in epithelial dysplasia. These include the following: loss of polarity of the basal cell (#1), the presence of more than one layer having a basaloid appearance (#2), drop-shaped rete-ridges (#4), irregular epithelial stratification (#5), and the presence of mitotic figures in the superficial half of the epithelium (#8). All of these may be caused by a solid proliferation of basaloid cells which are most primitive in the epithelial layer and are not differentiated to basal cells nor prickle (WHO, 1997). Also, there are 13 criteria of atypical features indicated by the WHO classification, however, they are neither always present nor all present at the same time in one lesion. Thus, it is not always clear what these findings mean in each case.

Recently, we reported that oral CIS has a definite tendency toward keratinization as one of its biological behavior, which is different from CIS of uterine cervix, in that basaloid cells mainly proliferate and do not show keratinization (Syafriadi et al., 2003). Also several histological subtypes of oral CIS such as CIS-basaloid, verrucous and acanthotic type (Syafriadi et al., 2006) have been reported and found in CIS of cervix uteri.

In this study, we analyze the other side of biological behavior of basaloid cell appearance of oral CIS using Ki-67 for cell proliferation pattern and TUNEL assay to study apoptotic cells. Many authors have used P53 protein tumor suppressor genes and Ki-67 as a parameter of cell proliferation in G1 phase to predict malignant transformation. They reported that P53 and Ki-67 protein over-expression were frequently found in both malignant and dysplastic lesion which increase due to grades of dysplasia, and may be an early event in multistage carcinogenesis of head and neck cancer (Kushner et al., 1997; Kovesi et al., 2003; Farrar et al., 2004). On the other hand, detection of apoptotic cells in the oral epithelium has been documented by using the TUNEL method (Okazaki et al., 2002; Abiko et al., 1994; Loro et al., 1999). As reported by Loro et al. in oral epithelium (Loro et al., 2003) and rat vaginal epithelium (Rao et al., 1998), TUNEL method may detect cell death not only by apoptosis but also through terminal differentiation towards keratinization (Loro et al., 2003). It is well-known that in apoptotic cells, DNA cuts into several nucleosomes. TUNEL method by using fluorescein-labeled dUTP and anti-fluorescein antibodies has been shown to be more sensitive to detect apoptotic cells than other TUNEL methods and equally accurate in comparison to other apoptosis detection methods (Duan et al., 2003).

In this study we used hyperplasia, mild and moderate dysplasia as control and for comparison in order to carry-out a definitive histopathological diagnosis and to know how they could replace whole layer of epithelium.

![Figure 1: Ki-67 immunostaining showing immunopositive cells dominantly located in suprabasal layer of hyperplastic (A) and mild dysplasia cases (B), when severity of dysplasia increased to moderate dysplasia, Ki-67 started involving basal layer (C) and more enhanced and increased in number when they transformed to CIS (D), that expressed until whole layer of CIS.](image)

**MATERIALS AND METHODS**

**Materials**

Seventy biopsies or surgical specimens from the oral mucosa were selected from the surgical pathology files in the Division of Oral Pathology, Niigata University Graduate School of Medical and Dental Sciences during a six year period from 1999 to 2004 after critical reviewing of hematoxylin and eosin (HE) stained sections. These consist of 20 cases of hyperplasia, 35 of epithelial dysplasia (mild, 20; moderate, 15), and 15 cases of CIS. The intra oral sites of the specimens taken were as follows: gingival, 10; tongue, 10; hard palate, 10; buccal mucosa, 15; soft palate, 5; oral floor, 20.
Three oral pathologists with the Japanese Society of Pathology board certification screened the specimens, when the diagnosis of grading of epithelial dysplasias are not identical, the case will be re-evaluated together. All of the specimens were routinely fixed in 10% formalin and embedded in paraffin.

After being washed with PBST, they were incubated with anti-fluorescein antibodies for 30 min at room temperature. The peroxidase reaction products were visualized by incubation with 0.02% 3, 3-diaminobenzidine (DAB, Dohjin Laboratories, Kumamoto, Japan) in 0.05 M Tris-HCl solution (pH 7.6) containing 0.005% H2O2. The sections were counter stained with haematoxylin. Cells were regarded as positive for Ki-67 staining and Tunel staining examined using light microscopy at 100 times magnification. The Ki-67 positive cells were counted in a square unit 1 mm² on a microscope equipped with a micrometer. Ten fields were randomly counted per section at x100 magnification. One-way ANOVA was used for statistical comparison of cell numbers between each group by using the SPSS software program (SPSS Inc., Chicago, IL, USA).

Methods

Serial 4 µm sections were cut from paraffin blocks. One set of the section was stained with haematoxylin and eosin and it was used for reevaluation of histological diagnosis, one slide used for Ki-67 immunohistochemistry and the other sets for Tunel examination. After deparaffinization and dehydration, sections were washed in 0.01 M phosphate buffered saline (PBS). To restore the antigenic sites, sections were autoclaved in 0.01 M citrate buffer (pH 6.0) for 15 min at 121˚C and then kept standing for 20 min at room temperature. To block endogenous peroxidase activities, all the sections were quenched with 0.001% H2O2 in 100% methanol for 30 min at room temperature and rinsed with PBS containing 0.5% skim milk and 0.05% triton X-100 (PBST). After rinsing in PBST, the sections were incubated in 5% skim milk in PBS containing 0.05% Triton X-100 (PBST). After incubation in PBST, the sections were incubated in 5% skim milk in PBS containing 0.05% Triton X-100 for 1 hr at 37˚C to block non-specific protein bindings. The sections were then incubated overnight at 4˚C with monoclonal primary antibodies against Ki-67 (1:100, Dako Ltd. Glostrup, Denmark). After incubations, the sections were washed in PBST and then treated with polymer-immune complexes (Envision+ peroxides, rabbit/mouse, Dako, 1:1) for 1 hr at room temperature. For Tunel examination, the slides were incubated with a TUNEL reaction mixture containing fluorescein-dUTP, dNTP, and terminal deoxynucleotidyl transferase (TdT) for 1 hour.

Figure 2: The expression of Ki-67 immunostaining showing immunopositive cells increased from hyperplastic, mild moderate and CIS cases, and statistically different each cases (p<.05).

Figure 3: H-E staining and Tunel immunostaining. Hyperplasia cases (A) showing apoptotic immunopositive cells dominantly located in superficial layer (B) whereas apoptotic cells in moderate dysplasia (C) (arrow) found in middle of layer (prickle area) (D), when severity of dysplasia increased to CIS (E,G) the apoptotic cells (arrow) found not only in the middle layer but also in basal layer (F). When the basaloivd cells replaced whole layer of epithelium (G) the apoptotic cells restricted only in superficial/keratin layer (H) seems like hyperplastic cases (arrow).
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RESULTS

Immunohistochemical staining for Ki-67, when observed, was found exclusively in the nuclei of epithelial cells. In the epithelial hyperplasia and mild dysplasia, Ki-67 immunopositive cells were dominantly located in supra/parabasal layer (Fig. 1A, B), when severity of dysplasia increased to SED-moderate, Ki-67 started involving basal layer (Fig. 1C) and more enhanced and increased in number when they transformed to CIS (Fig. 1D), that expressed until whole layer of CIS. There were statistically significant differences of Ki-67 expression between hyperplasia, mild dysplasia, moderate dysplasia and CIS (p<0.05) (Fig. 2).

Immunohistochemical staining for apoptotic detection showed that hyperplastic (Fig. 3A), apoptotic cells, were located only on the surface keratinized layers (Fig. 3B). However, in moderate dysplasia (Fig. 3C) apoptotic cells concentrated in the middle layer of the dysplastic epithelium (Fig. 3D). However, if severity of dysplastic were increased (Fig. 3E), apoptotic cells were observed not only in the middle layer but also in the basal layer (Fig. 3F) therefore, when whole layers were totally occupied with basaloid cells proliferation (Fig. 3G), apoptotic cells were restricted to the surface layer (Fig. 3H).

DISCUSSION

It is important to identify basaloid cells formation in intra epithelial of oral CIS because based on WHO classification, the presence of more than one layer having a basaloid appearance is one of the criteria of an atypical cell (WHO, 1997). The basaloid cells in oral CIS may have similarity function with basal cell in normal epithelium/normal keratinocytes. It is known that basal cells are a source for squamous epithelial regeneration and they are undifferentiated cells. In oral CIS we observed that basaloid cells have same function as normal basal cell of oral epithelium but they are carcinomatous cells. It has been generally accepted that some of the oral squamous cell carcinomas (SCC) develop through their precancerous steps of squamous epithelial dysplasia and CIS (Lumerman et. al., 1995).

Figure 4: The behavior of basaloid cell appearance from hyperplasia/mild dysplasia to carcinoma in-situ sequence is schematically shown above, which is basaloid cells initially found in parabasal layer (a) and slowly expanded to basal direction resulted basal cell alignment (b) and to prickle layer direction (c) and resulted “two-phase appearance” (basaloid cells in the lower part and keratinization layer in upper part). When they transformed to CIS the basaloid cells were take over until whole layer of epithelium (d).
Using Ki-67 marker proliferation, in this study hyperplasia cases show that most cells with proliferating potential (immunopositive staining cells) are located in parabasal or suprabasal layer (Fig. 1B). It is not basal cells which may be regarded as undifferentiated cells or germinal cells of the oral squamous epithelium. The basal cells can be considered as terminally-differentiated cells. Saku et al., reported basal layer of epithelial hyperplasia is immunopositive for keratin 19 markers implying that basal cells are differentiated cells (The Japanese Society for Oral Pathology, 2005).

In mild and moderate epithelial dysplasia cases, the number Ki-67 immunopositive basaloid cells is increased concomitantly with severity of dysplasia (Fig. 1B,C,D and Fig. 2), but in moderate dysplasia, Ki-67 immunopositive basaloid cells are not only found in suprabasal layer but also in basal layer and prickle layer (Fig. 1C). There seems to be a stepwise of invasive development, even though basement membrane is still preserved. In the upper layer or surface of mild and moderate dysplasia, differentiation and keratinization cells are definitely found, but when basaloid cells replaced whole layer of epithelium such as called CIS, the Ki-67 immunopositive basaloid cells are found sporadically in basal, suprabasal, prickle and surface layer (Fig. 1D). The summary of epithelial dysplasia to carcinoma in-situ sequence is schematically shown in figure 4.

Interestingly, since most of oral squamous cell carcinomas are observed to be well-differentiated, it is reasonable to propose that the basaloid cells are carcinomatous cells in intraepithelial of CIS, and after being proliferated they had capability toward differentiation and keratinization.

In the present study, we demonstrate that the apoptotic cells increase in number with the severity of epithelial dysplasia up to moderate dysplasia and then decrease in carcinoma in-situ when basaloid cells expand to whole layer epithelium. Therefore, the accumulation of apoptotic cells is found in the middle layer of dysplastic epithelium and some in basal layer (Fig. 2D, F). We propose that apoptosis in the basal and prickle area could allow basaloid cells to expand until surface of epithelium in CIS cases (Fig. 1D). The increase of apoptotic cells in epithelial dysplasia has earlier been reported by many authors (Kohn et al., 2002; Ravi et al., 1999; Birchall et al., 1995). Macluskey et al. (Macluskey et al., 2000) showed increasing tendencies of apoptosis towards the progression of oral squamous cell carcinoma.

From this study, it can be concluded that basaloid cells in epithelial dysplasia and CIS can be detected using Ki-67 as proliferating cell marker and those cells seemingly located in parabasal layer, otherwise, basal layer are differentiated cells. The proliferating cells called basaloid cells could be regarded as carcinomatous cells, and could replace whole epithelium due to many cells have apoptosis in basal and prickle layer. Therefore, these basaloid cells have definite evidence towards differentiation and keratinization. In the future further studies are necessary to examine the relationship between apoptosis and basaloid formation and their way to extend and to replace whole layer of intra epithelium.

References


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