

# Expression of CD40 and Fas Ligand in Bowen's Disease, Squamous Cell Carcinoma and Basal Cell Carcinoma

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Impaired regulation of apoptosis is known to be associated with the development of various cancers, and Fas/Fas-ligand (FasL) is known to play an important role in apoptosis. CD40 is a cell surface receptor, which when ligated modulates apoptosis in some cell types. The expressions of CD40 and FasL were examined in 10 normal skins, 7 Bowen's disease skins, 10 squamous cell carcinomas (SCCs) and 12 basal cell carcinomas (BCCs) immunohistochemically. In the normal epidermis, CD40 was more highly expressed in the keratinocytes of the squamous cell and granular layers than in those of the basal layer, and FasL expression was observed in the cell membrane of keratinocytes at the basal and squamous cell layers. CD40 expression was significantly higher in SCCs than in normal or Bowen's disease skin, while FasL expression was significantly higher in Bowen's disease than in SCCs. BCCs expressed the lowest levels of CD40 and FasL. These results suggest that altered CD40 and FasL expression may be related with the progression of SCC, and the marked reduced expression of CD40 and FasL may explain the biologic behavior of BCCs.

**Key Words:** CD40, FasL, Bowen's disease, squamous cell carcinoma, basal cell carcinoma

## INTRODUCTION

CD40 is a cell surface molecule belonging to the tumor necrosis factor (TNF) receptor group of molecules, which are involved in the transmission

of apoptotic/anti-apoptotic signals in various types of cells.<sup>1</sup> In B lymphocytes, CD40 activation antagonized CD95-mediated apoptosis.<sup>2</sup> However, on other cell types expressing CD40, such as epithelial cells, its effect is not clearly defined.<sup>3</sup> CD40 activation in bladder, pancreatic and breast carcinomas, as well as melanoma cell lines showed significant proliferation inhibition due to alterations in the cell cycle and apoptotic induction.<sup>4</sup> Decreased CD40 expression has been reported in squamous cell carcinoma (SCC)<sup>5,6</sup> and basal cell carcinoma (BCC),<sup>5</sup> and has been suggested to promote tumor progression. In contrast, CD40 showed anti-apoptotic effects in nasopharyngeal carcinoma cells<sup>7</sup> and prostatic carcinoma cell lines<sup>8</sup> like B lymphocytes. This finding seems to be closely associated with the progression of malignant melanoma and lung cancer.<sup>9,10</sup>

Fas ligand (FasL) belongs to the TNF superfamily of membrane and secreted proteins.<sup>11</sup> The Fas/FasL system plays an important role in apoptosis,<sup>12</sup> and FasL expression was first considered to be restricted to activated T lymphocytes and natural killer cells.<sup>13</sup> However, the identification of FasL expression in immune-privileged sites,<sup>14,15</sup> such as the stromal cells of the eye and the Sertoli cells of the testis, suggested that FasL may be important for maintaining immune privilege. Recent studies have found that several human tumors show the up-regulated FasL expression, which is able to kill the Fas-bearing cell.<sup>16-18</sup>

I hypothesized that altered CD40 and FasL expressions may be related to the progression of SCC and explain the biologic characteristics of BCC. Therefore, I examined by immunohistochemistry the expression of CD40 and FasL in normal skin, Bowen's disease, SCC and BCC.

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## MATERIALS AND METHODS

### Tissue samples

Seven Bowen's disease skin samples, 10 SCCs and 12 BCCs tissue samples were obtained from the Pathology Department of Dongguk University College of Medicine, Kyongju, Korea. The SCCs were graded histologically according to the proportion of differentiated cells, and consisted of 4 well differentiated, 3 moderately differentiated and 3 poorly differentiated tumors. Ten normal skins were obtained from the epidermal cyst lesion samples.

### Immunohistochemistry

Sections of 4- $\mu$ m thickness were prepared from formalin fixed and paraffin embedded tissue blocks and spread on poly-L-lysine coated slides. The sections were immersed in three changes of xylene and hydrated using a graded series of alcohol solutions. Antigen retrieval was performed routinely by immersing the sections in 0.01 M citrate buffer (pH 6.0) in a pressure cooker and autoclaving for 15 min. After rinsing with phosphate-buffered saline (PBS), the sections were treated with 1% H<sub>2</sub>O<sub>2</sub> in PBS for 15 min at room temperature to abolish endogenous peroxidase activity. After washing with PBS, the slides were incubated with primary antibody for 1 hr at room temperature. The primary antibodies used were:- rabbit polyclonal anti-human CD40 (N-16, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1: 100, and rabbit polyclonal anti-rat FasL (N-20, Santa Cruz Biotechnology) diluted 1:200. Normal rabbit IgG was used as a negative control. Staining was performed using a DAKO LSAB+kit (DAKO, Carpinteria, CA, USA) and developed with 3, 3'-diaminobenzidine tetrahydrochloride (Zymed Laboratories, Inc., San Francisco, CA, USA). The sections were counterstained for 5 min with Meyer's hematoxylin and then mounted. The intensity of the signal was graded as follows:- 0, absent; 1, mild; 2, moderate; 3, severe. The area of the signal was recorded as:- 0, no expression; 1, 1-25% positive cells; 2, 26-49% positive cells; 3, >50% positive cells. The expression index was calculated as the

sum of intensity and area.

### Statistical analysis

Group comparisons of the expression indices of CD40 and FasL were performed using Kruskal-Wallis one-way ANOVA and the Mann-Whitney U-test.  $p < 0.05$  was considered significant.

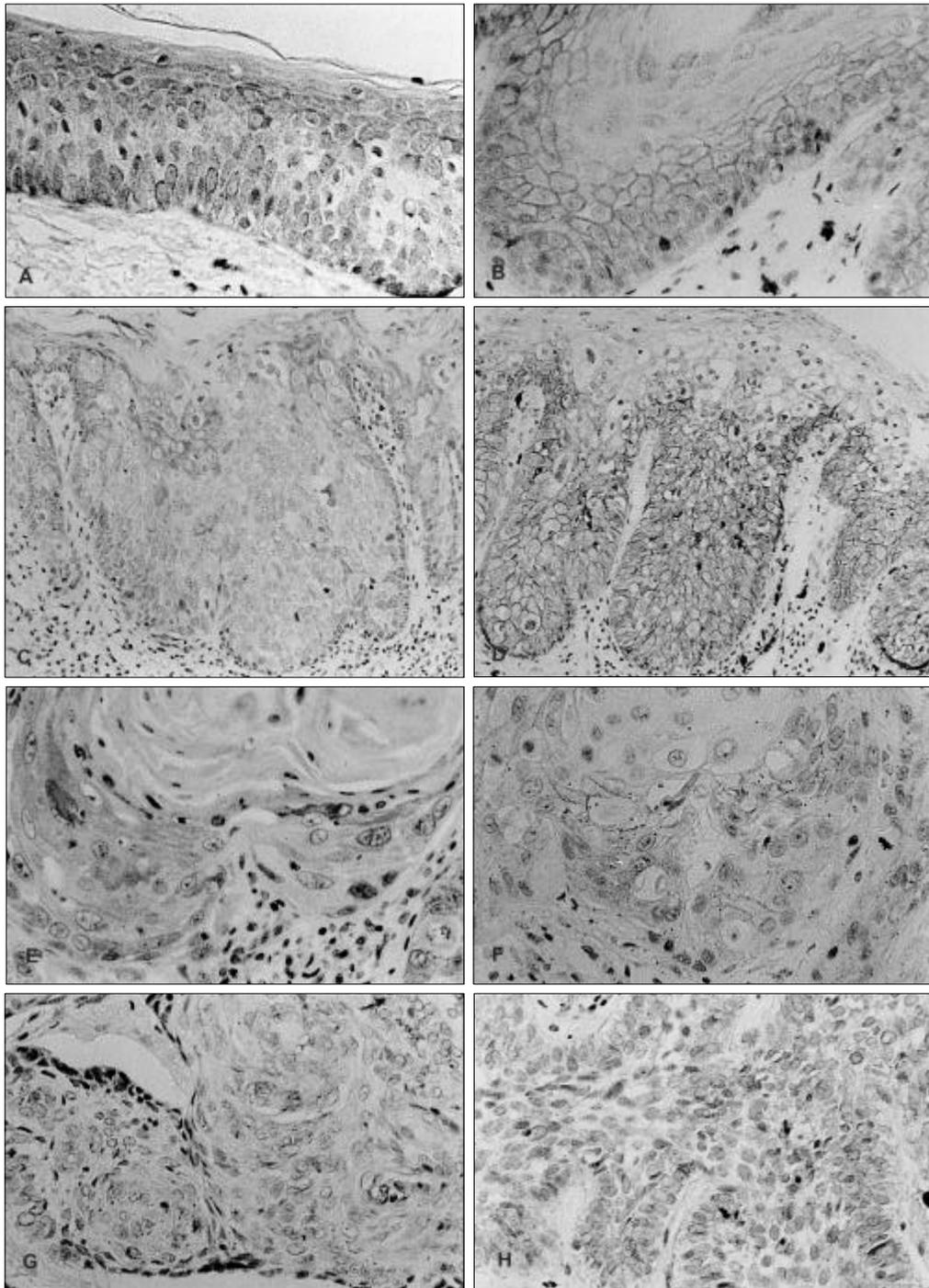
## RESULTS

### Expression of CD40

In normal skin, CD40 was expressed on the cytoplasm of all keratinocytes, but its staining intensity was higher in the squamous cell and granular layers than in the basal layer (Fig. 1A). In Bowen's disease, CD40 expression predominated in tumor cells with abundant cytoplasm, and was undetectable in tumor cells with little cytoplasm (Fig. 1C). In SCC, CD40 was not expressed in the keratinized squamous tumor cells located near the center of tumor nests (Fig. 1E), however no correlation was found between CD40 expression and the grade of differentiation. As shown in table 1, the CD40 expression index was significantly higher in SCC than in normal skin, Bowen's disease or BCC ( $p < 0.05$ ). BCC had the lowest CD40 expression index, and in 3 cases CD40 was not expressed ( $p < 0.05$ ) (Fig. 1G) (Table 1).

### Expression of FasL

In normal skin, FasL expression was observed in the cell membrane of keratinocytes at the basal layer and the squamous cell layer (Fig. 1B). In Bowen's disease, the expression pattern of FasL was similar to that of normal skin (Fig. 1D), and its expression index significantly higher than those of SCC and BCC (Table 1). In SCC, FasL was expressed in the nonkeratinized squamous tumor cells located at the periphery of tumor nests (Fig. 1F), however no correlation was found between FasL expression and the grade of differentiation. BCC had the lowest FasL expression index, and was not expressed in 4 cases ( $p < 0.05$ ) (Fig. 1H) (Table 1).



**Fig. 1.** Immunohistochemical staining of CD40 (A, C, E, G) and Fas ligand (B, D, F, H) in normal skin (A, B), Bowen's disease (C, D), squamous cell carcinoma (E, F) and basal cell carcinoma (G, H). In the normal epidermis, CD40 was expressed more so in the keratinocytes of the squamous cell and granular layers than in the basal layer, whereas, FasL expression was observed in the cell membranes of keratinocytes located at the basal and squamous cell layers. In Bowen's disease, CD40 expression predominated in tumor cells with large cytoplasm, and was undetectable in tumor cells with a small cytoplasm, and in addition, the pattern of FasL expression was similar to that of normal skin. In squamous cell carcinoma, CD40 and FasL were expressed in the non-keratinized cancer cells located at the periphery of tumor nests, but not in the keratinized tumor cells located near the center of tumor nests. Basal cell carcinoma expressed the lower levels of CD40 and FasL than normal skin, Bowen's disease and squamous cell carcinoma. Magnification: A, B and E-H,  $\times 200$ ; C and D,  $\times 100$ .

**Table 1.** Expression Index of CD40 and FasL in Normal Skin, Bowen's Disease, Squamous Cell Carcinoma and Basal Cell Carcinoma

	Number of cases	CD40	FasL
Normal skin	10	4.49 ± 0.76	4.86 ± 0.69
Bowen's disease	7	4.29 ± 0.76	5.57 ± 0.54 <sup>‡</sup>
Squamous cell carcinoma	10	5.50 ± 0.85 <sup>*</sup>	4.70 ± 0.82
Basal cell carcinoma	12	3.08 ± 1.93 <sup>†</sup>	2.33 ± 1.06 <sup>†</sup>

Values are means ± SD. Statistical significance was determined using the Mann-Whitney U-test.

<sup>\*</sup>*p* value < 0.05 relative to normal skin, Bowen's disease and basal cell carcinoma, <sup>†</sup>*p* value < 0.05 relative to normal skin, Bowen's disease and squamous cell carcinoma, <sup>‡</sup>*p* value < 0.05 relative to squamous cell carcinoma and basal cell carcinoma.

## DISCUSSION

This study demonstrated that CD40 immunoreactivity was significantly higher in SCC than in Bowen's disease and normal skin. CD40 is an important T and B lymphocyte stimulatory molecule, and is involved in cell survival, activation, differentiation and anti-apoptosis.<sup>2,19</sup> CD40 is also expressed in various kinds of non-immune cells such as epithelial cells, mesenchymal cells and endothelial cells.<sup>3</sup> Recent studies have shown that several types of malignant cells, including melanoma and carcinomas of bladder, colon, prostate, breast and lung, express CD40.<sup>4,9</sup> However, the effects of CD40 activation are not known in epithelial cells or various kinds of carcinomas. CD40 has anti-apoptotic activity in nasopharyngeal carcinoma cells<sup>7</sup> and prostatic carcinoma cell lines,<sup>8</sup> and CD40 expression is closely associated with the progression of malignant melanoma and lung cancer.<sup>9,10</sup> These findings support our results. However, Viac et al.<sup>5</sup> reported the attenuated CD40 expression in SCC, and suggested that this might help the tumor cells escape from immunosurveillance. In addition, it has been shown that CD40 expression is lower in SCC than in Bowen's disease.<sup>6</sup> This discrepancy may have been caused by different antibodies used. However, no satisfactory explanation for this discordance has been given. The *in vivo* function of CD40 in skin squamous neoplastic lesions remains to be determined.

Lee et al.<sup>20</sup> reported that FasL is strongly expressed in the granular layer. However, in the present study, normal skin showed FasL expression in the basal and squamous cell layers but not in the granular layer. As has been shown, the

normal esophagus expresses FasL in the basal and suprabasal layers.<sup>18</sup> Therefore, FasL expression may protect basal keratinocytes from apoptotic cell death by inducing the Fas-mediated apoptosis of activated lymphocytes. Although no correlation was found between FasL expression and the grade of differentiation in SCC, FasL was expressed mainly in the nonkeratinized squamous tumor cells located at the periphery of tumor nests. Moreover, the apoptotic deletion of tumor-infiltrating lymphocytes was observed more frequently in FasL-positive areas than in FasL-negative areas.<sup>21</sup> Therefore, it appears that carcinoma cells in the peripheral zone of tumor nests may escape apoptosis by killing lymphocytes through Fas-mediated apoptosis. Unexpectedly, FasL expression was lower in SCC than Bowen's disease, but on the other hand, CD40 expression was higher in SCC than in Bowen's disease. In the case of SCC, we suggest that CD40 may have an important anti-apoptotic role.

Interestingly, CD40 and FasL expression were lowest in BCC, which may make it hard for BCC cells to escape immunosurveillance. This finding may explain why BCC rarely metastasize; the incidence of BCC metastasis has been reported to be 0.0028%,<sup>22</sup> and which is in stark contrast to metastatic SCC, showing lymphatic metastasis in 80-90% of cases. Lymph nodes contain immunocompetent cells, which makes it difficult for tumor cells to survive. We believe it likely that the low incidence of BCC in the lymph nodes may also be explained by the reduced expression of CD40 and FasL.

In conclusion, this study suggests that altered CD40 and FasL expression may be related to the progression of SCC, and in addition, that mark-

edly reduced expression of CD40 and FasL in BCC may explain aspects of its biologic behavior.

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