Short communication

Immunohistochemical demonstration of p73 protein in the early stages of DMBA-induced squamous-cell carcinogenesis in hamster buccal pouch

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Abstract

The identification of a new protein, p73, with structural and functional similarities to p53 protein suggests that a family of p53-like proteins is likely to exist. This study investigated the status of p73 protein in the early stages of 7,12-dimethyl benz[a]anthracene (DMBA)-induced carcinogenesis. Outbred young (6-week-old) male Syrian golden hamsters (Mesocricetus auratus; 40 animals) were randomly divided into four equal groups: a 3-week DMBA-treated experimental group, a 6-week DMBA-treated experimental group, a mineral oil-treated control group, and a non-treated control group. Following this, a total of 80 specimens of pouch mucosa were obtained from the 40 animals in the four groups. Positive nuclear staining for p73 protein was randomly distributed throughout the whole epithelial layer of the DMBA-treated specimens and was absent in controls. Positive p73 staining was observed in 8 of the 20 (40%) 3-week, and 14 of the 20 (70%) 6-week DMBA-treated specimens. None of the 3-week DMBA-treated specimens revealed more than 25% p73-positive keratinocytes, but, in 12 (60%) of the 6-week-treated specimens, more than 25% of the keratinocytes examined were p73-positive. This suggests that the longer the DMBA painting period, the higher the proportion of p73-stained pouch keratinocytes. Furthermore, a p73-dependent mechanism may be associated with the early stages of oral carcinogenesis. Such a mechanism could be very important to an understanding of the participation of p73 in the development of oral squamous-cell carcinomas.

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It was thought that p53 was encoded by a gene lacking any close relatives and the existence of a p53 homologue had long been considered unlikely (Kaghad et al., 1997). In 1997, however, this viewpoint changed with the identification of a human homologue of p53, which was termed p73 (Kaghad et al., 1997). The p73 gene, located on chromosome 1p36.33, encodes a nuclear protein with significant homology to p53 (Kaghad et al., 1997). New evidence has now indicated that there exists a p53 gene family (Kaelin, 1999) and that p73 is one of its members. The p73 protein has substantial structural similarity to p53 in all three domains.

The NH1-terminal trans-activation domain (29% identity with p53 amino acids), the sequence-specific DNA-binding region (63% identity with p53) and the oligomerization region (38% identity with p53) are remarkably conserved between the two proteins (Kaghad et al., 1997).

The p73 can, at least when overproduced, activate the transcription of p53-responsive genes and inhibit cell growth in a p53-like manner by inducing apoptosis (Jost et al., 1997), but unlike p53, no mutant p73 has been found amongst human tumours (Nomoto et al., 1998; Yokozaki et al., 1999). In contrast, mutations in the p53 gene are detected in more than 50% of many types of cancers (Harris and Hollstein, 1993), and in 69% of human head-and-neck squamous-cell carcinomas (Ahmaddehbe et al., 1995).

Immunohistochemical expression of p73 protein has recently been reported in human oral squamous-cell carcinoma (Furudomi-Laurens et al., 2001), suggesting that this
p53-related protein may be associated with the late stages of oral cancer. Thus, we have now investigated whether or not the p73 protein could also participate in the early stages of oral carcinogenesis.

The hamster buccal pouch mucosa is one of the most widely accepted experimental models of oral carcinogenesis (Gimenez-Conti and Slaga, 1993). Despite anatomical and histological differences between pouch mucosa and human buccal tissue, experimental carcinogenesis in the hamster cheek pouch produces premalignant changes and carcinomas that resemble the development of premalignancy and malignancy in human oral mucosa (Morris, 1991). To understand better the status of p73 in oral carcinogenesis, we have studied the immunohistochemical expression of p73 protein in the early stages of DMBA-induced squamous-cell carcinogenesis in the hamster buccal pouch.

Outbred young (6-week-old) male Syrian golden hamsters (Mesocricetus auratus) (40 animals; purchased from the National Science Council Animal Breeding Centre, Taipei, ROC), weighing approximately 100 g at the beginning of the experiment, were randomly divided into two experimental groups (each containing 10 animals) and two control groups (each containing 10 animals). The animals were housed under constant conditions (22°C; 12 h light/dark cycle) and fed with tap water and standard Purina laboratory chow ad libitum. Appropriate care and an approved experimental protocol ensured humane treatment of the test animals; all procedures were conducted in accordance with the NIH Guide for the Care and Use of Animals.

After allowing the animals a week of acclimatisation to their new surroundings, both pouches in all animals of the experimental groups were killed simultaneously with a lethal dose of diethyl ether at 9 a.m. to the animals in one of the experimental groups were killed untreated throughout.

Both pouches at each painting. The other control group was similarly treated with mineral oil. Approximately, 0.2 ml of solution at 9 a.m. on Monday, Wednesday and Friday of each week, using a number four sable-hair brush. Both pouches were considered positive when more than 10% of the epithelial cells proved to be positive (scores 2 and 3). Thicker sections of each specimen were cut at 4 μm thickness. One of the sections was prepared for the p73 immunohistochemical study, the other was used for haematoxylin–eosin staining.

Immunohistochemical staining was done by a standard ABC method (Hsu et al., 1981). Rabbit polyclonal antibodies to p73, obtained from Santa Cruz Biotechnology Inc. (cat. no. sc-7957), had been raised against a recombinant protein corresponding to amino acids 1–80 mapping at the amino terminus of human-origin p73. According to the manufacturer, this antibody recognises all p73 isoforms of human, rat and mouse origin by Western blotting, immunoprecipitation and immunohistochemistry. After deparaffinization in xylene and ethanol, the sections were treated with 0.3% H2O2–methanol and 10% normal goat serum (Dako, Santa Barbara, CA, USA). All sections were subsequently incubated with primary antibody (1:200) at room temperature for 30 min. They were then incubated for a further 30 min at room temperature with biotin-conjugated goat antirabbit IgG (1:100; Vector, Burlingame, CA, USA) and then for a final 30 min with ABC (Dako). The sites of peroxidase binding were visualised as brown reaction products by the diaminobenzidine reaction. The sections were counterstained with haematoxylin. Each set of experiments included a specimen of human buccal squamous-cell carcinoma known to express p73 to serve as a positive control. The proportion of positively stained cells expressing only cytoplasmic staining were excluded. Therefore, only the proportion of cells demonstrating nuclear expression of p73, not the associated intensity of histochemical staining (Hall and Lane, 1994), was quantified. The proportion of positively stained cells in the whole layer of epithelium was determined by observing the entire section. The scoring system used was as follows: 3—more than 25%; 2—10–25%; 1—<10% of epithelial cells scoring positive; 0—no epithelial cells stained. Pouches were considered positive when more than 10% of the epithelial cells proved to be positive (scores 2 and 3).

Gross examination of all of the mineral oil-treated and untreated pouches revealed no apparent changes. Thicker mucosa with a rough surface and whitish granular appearance was observed in the 3- and 6-week DMBA-treated pouches. Occasional erythematous areas were also observed in the DMBA-treated mucosa.

No significant histological changes were observed in any of the mineral oil-treated and untreated pouches (Fig. 1), whereas hyperkeratosis and epithelial dysplasia were found in the 3- and 6-week DMBA-treated pouches (Fig. 2A and B).

The results of the immunostaining are summarised in Table 1. The presence of p73 as indicated by positive staining was not revealed in all of the keratinocytes of the DMBA-treated specimens. Within those specimens, strong
Fig. 1. Representative section of untreated pouch mucosa; negative for p73 nuclear staining. ABC stain; 40×.

nuclear immunoreactivity was generally observed; with a few exceptions, the cytoplasm was not immunoreactive. Only in a very small number of DMBA-treated specimens that had strong nuclear staining was a faint intracytoplasmic immunoreactivity observed. Furthermore, cells exhibiting positive nuclear staining were randomly distributed throughout the whole layer of pouch mucosa (Fig. 2A and B). Positive p73 staining was observed in 8 of the 20 (40%) 3-week (Fig. 2A) and 14 of the 20 (70%) 6-week (Fig. 2B) DMBA-treated specimens. None of the 3-week specimens

Fig. 2. Nuclear positivity for p73 located randomly within the entire epithelium in representative sections of (A) 3-week and (B) 6-week DMBA-treated pouch mucosa. ABC stain; 100×.
oral carcinogenesis in concert with p53 and/or p21 (Jost et al., 1997), the (potential) participation of p73 in the early stages of oral carcinogenesis could be very important in the overall understanding of the role of p73 in the early stages of oral carcinogenesis. Such a mechanism may be associated with a p73-dependent mechanism may be associated with the outcome of the current study suggests that a p73-dependent mechanism could be important in the overall understanding of the participation of p73 in the development of oral squamous-cell carcinomas.

Acknowledgements

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Table 1

<table>
<thead>
<tr>
<th>DMBA painting period</th>
<th>Total number of pouch mucosa specimens</th>
<th>Scores for p73 staining (number of cases)</th>
<th>Positive rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Score 0</td>
<td>Score 1</td>
</tr>
<tr>
<td>3 weeks</td>
<td>20</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>6 weeks</td>
<td>20</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>No painting</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mineral oil painting</td>
<td>20</td>
<td>20</td>
<td>0</td>
</tr>
</tbody>
</table>

Scores for p73 staining (number of cases) Positive rate (%)

Table 1 Immunostaining of p73 in the early stage of DMBA-induced hamster buccal pouch squamous-cell carcinogenesis

Exhibited more than 25% p73-positive keratinocytes, but in 12 (60%) of the 6-week specimens, more than 25% of the keratinocytes were p73 positive. Neither mineral oil-treated nor untreated specimens exhibited p73 positivity. The negative control omitting the primary anti-p73 antibody demonstrated no definitive staining; the positive control revealed distinct positive immunostaining for the p73 protein.

The p73 protein has recently been demonstrated in a subset of head-and-neck squamous-cell carcinomas, including oral carcinomas (Faridoni-Laurens et al., 2001). In the current study, using the hamster buccal pouch model, we detected the immunorepression of p73 protein in the early stages of DMBA-induced experimental oral carcinogenesis. The longer the DMBA painting period, the higher the proportion of p73-stained pouch keratinocytes (Table 1). To the best of our knowledge, this is the first study describing the expression of p73 protein in the early stages of experimental oral carcinogenesis. Furthermore, despite an intensive search of the available literature, no mutation of the p73 protein has been found in human cancers (Kaghad et al., 1997; Maet al., 1998; Nonoto et al., 1998; Takahashi et al., 1998; Yang et al., 1999), including oral carcinomas (Faridoni-Laurens et al., 2001). Judging from these results, it seems likely that wild-type, not mutant, p73 was overexpressed in the present study, suggesting that p73 could play an important part in oral carcinogenesis through the overexpression of the wild type rather than as a tumour suppressor.

On the other hand, due to the remarkable homology between p73 and p53, together with the ability of p73 to induce the expression of p21/WAF1/CIP1 (Clurman and Groudine, 1997; Jost et al., 1997), the (potential) participation of p73 in oral carcinogenesis in concert with p53 and/or p21/WAF1/CIP1 should not be overlooked: the disruption of normal p53 function may result in a compensatory upregulation of p73 expression; and, either mutant p53 or the reduction of p21/WAF1/CIP1 may also trigger an increase in p73 expression.

Further study of the association between the p53 mutation and p73 overexpression is required to comprehend fully the role of p73 in the early stages of oral carcinogenesis.

In conclusion, regardless of which mechanism is implicated, the outcome of the current study suggests that a p73-dependent mechanism may be associated with the early stages of oral carcinogenesis. Such a mechanism could be very important in the overall understanding of the participation of p73 in the development of oral squamous-cell carcinomas.

References


