E-Cadherin Expression in Squamous Cell Carcinomas of Head and Neck: Inverse Correlation with Tumor Dedifferentiation and Lymph Node Metastasis

Jörg H. Schipper, Uwe H. Frixen, Jürgen Behrens, Andreas Unger, Klaus Jahnke, and Walter Birchmeier

Institut für Zellbiologie (Tumorforschung) [J. H. S., U. H. F., J. B., W. B.], and Department of Otorhinolaryngology [J. H. S., A. U., K. J.], Medical School, University of Essen, 4300 Essen 1, Germany

ABSTRACT

Tissue sections of 32 squamous cell carcinomas (SCCs) of the head and neck were investigated for the expression of the epithelium-specific cell adhesion molecule E-cadherin. We found that E-cadherin expression is inversely correlated both with the loss of differentiation of the tumor and with lymph node metastasis. The well-differentiated SCCs expressed E-cadherin, often as strongly as the normal stratified epithelium (12 cases were tested); the moderately differentiated SCCs expressed intermediate amounts of E-cadherin or were heterogeneous (15 cases were analyzed); whereas the poorly differentiated SCCs were all E-cadherin-negative (five cases were investigated). Furthermore, seven of eight infiltrated lymph nodes of SCCs were E-cadherin-negative. These data indicate that the loss of the cell adhesion molecule E-cadherin in fact plays an important role in the progression of human squamous cell carcinomas, i.e., that down-regulation of expression is associated with dedifferentiation and metastasis of the tumor cells in vivo.

INTRODUCTION

Head and neck carcinomas of the upper aerodigestive tract are the sixth most frequent human tumors diagnosed worldwide, and their tendency is increasing (1–3). Roughly, three levels of tumor locations are recognized, the nasopharynx, the pharynx (including floor of mouth, oropharynx, and hypopharynx), and the larynx. The carcinomas originate in the mucosa which lines the inner surfaces of the cavities of the aerodigestive tract, and 80–90% are SCCs, followed by adenocarcinomas and others (3, 4). According to the WHO, squamous cell carcinomas of head and neck can be subdivided by grade of epithelial differentiation into well, moderately, and poorly differentiated types (2, 3, 5). It has been suggested that these grades of differentiation are associated with patient survival, whereby lower differentiation means shorter survival (2, 6). Little is known about risk factors and the multistep process of tumorigenesis in SCCs of the head and neck; nicotine and alcohol abuse play a role (1, 3, 7). Furthermore, human papillomavirus and Epstein-Barr virus involvement has been reported for nasopharynx and larynx carcinomas, respectively (8, 9). Recently, amplification, overexpression, and mutation of a number of oncogenes have been described in head and neck cancer (10–17). An important factor for the success of clinical therapy of SCCs of the head and neck is the state of the involvement of lymph node metastasis (2, 18). In this process, carcinoma cells detach from the primary tumor mass, migrate through the surrounding connective tissue, invade neighboring lymphoid vessels, and colonize the nearest lymph nodes. In undisturbed squamous epithelia this does not occur, since the individual cells are interconnected by a complex network of cell-cell adhesions. Only the disruption of such mutual interactions may allow the carcinoma cells to migrate away from the primary tumor (19, 20).

Our laboratory has previously studied epithelial differentiation and invasion in vitro with respect to the expression and function of the epithelium-specific cell-cell adhesion molecule E-cadherin. We have demonstrated that nontransformed Madin-Darby canine kidney epithelial cells acquire invasive properties when intercellular adhesion is specifically inhibited by the addition of antibodies against E-cadherin; the separated cells then assume a fibroblast-like (i.e., dedifferentiated) morphology and invade collagen gels and embryonal heart tissue. Furthermore, epithelial cells transformed with Harvey and Mo-loney sarcoma viruses were found to be constitutively fibroblast-like invasive, and they do not express E-cadherin (21). We also confirmed this correlation by examining various human cell lines derived from bladder, breast, lung, and pancreas carcinomas. We found that carcinoma cell lines with an epithelioid phenotype were noninvasive and expressed E-cadherin, whereas carcinoma cell lines with a fibroblastoid phenotype were invasive and had lost E-cadherin expression. Invasiveness of dedifferentiated breast carcinoma cells could be prevented by transfection with E-cadherin cDNA and was again induced by treatment of the transfected cells with anti-E-cadherin monoclonal antibodies (22). These findings indicate that the selective loss of E-cadherin expression can generate dedifferentiation and invasiveness of human carcinoma cells in vivo.

In this study we have investigated the expression of the cell-cell adhesion molecule E-cadherin in SCCs of the head and neck and have assessed the results with histopathological differentiation and lymph node metastasis.

MATERIALS AND METHODS

After clinical staging and biopsy, squamous cell carcinoma tissue of the head and neck was surgically removed from previously untreated patients. Lymph nodes were removed by neck dissection. The material was immediately frozen in liquid nitrogen. A separate portion was used for routine histopathological grading by the Department of Pathology, University of Essen Medical School. Frozen tissue sections were prepared in a Frigocut 2800-E microtome (Reichert-Jung, Nussloch, Germany). The tumor tissue was initially localized by staining with toluidine blue, and then serial sections of 6 μm were cut and stained for immunofluorescence (after ethanol fixation at −20°C) by in situ hybridization (after formaldehyde fixation) and with hematoxylin or hematoxylin/eosin. Infiltrated lymph nodes were sectioned and stained in a similar manner, except that 10-μm sections were used.

Immunofluorescence staining of primary tumors, infiltrated lymph nodes, and cell lines of SCCs was performed with the anti-E-cadherin monoclonal antibody 6F9 as described previously (21, 22) or with a rabbit polyclonal anti-pancytokeratin antibody (K40, a generous gift of Dr. F. Ramaekers, Maastricht, The Netherlands). As second antibodies, fluorescein isothiocyanate-conjugated goat anti-mouse and rhodamine isothiocyanate-conjugated goat anti-rabbit IgG (Dianova) were used. In situ hybridizations were performed as previously described (23, 24). Briefly, antisense and sense riboprobes of a 386-base pair human E-cadherin cDNA were prepared by in vitro transcription with T7 RNA polymerase from a plasmid having a T7 promoter region. The probes were nick-translated with [γ-32P]ATP using T4 polynucleotide kinase and hybridized overnight at 42°C to 30–50 μg of denatured, sonicated SCC DNA extracted as previously described (25).
cadherin cDNA (HC6-1; Ref. 22) in the Bluescript vector were produced by T3 and T7 RNA polymerase (Stratagene), respectively, using $^{35}$S-labeled UTP and CTP (Amersham). Hybridization of sections was performed at 47°C in 50% formamide-2× SSC-5 mM EDTA-10 mM dithiothreitol-10 mM 2-mercaptoethanol-10 mM Tris-HCl, pH 7.5, -10 mM NaH$_2$PO$_4$, pH 6.8, containing 150 μg/ml salmon sperm DNA and 10 μM S-ATP. Sections were washed with 50% formamide-2× SSC for 2 h and then with 50% formamide-0.1× SSC for 12 h. Slides were dipped into Kodak NTP-2 emulsion diluted 1:1 and exposed for 7–14 days at 4°C. For Western blot analysis of tissue extracts, 25 frozen sections of 20 μm were extracted for 30 min at 4°C with 3% Triton X-100 in L-CAM assay buffer-100 mM phenylmethylsulfonyl fluoride, and the extracts were processed as described previously (21, 22).

**RESULTS**

**E-Cadherin Expression in SCC Cell Lines and Tissues of Head and Neck.** Well-, moderately, and poorly differentiated SCC cell lines were examined for E-cadherin expression by both immunofluorescence and Western blotting. A well-differentiated cell line expressed E-cadherin, whereas the moderately and poorly differentiated lines were negative (Figs. 1 and 2).

In the main part of this work, E-cadherin expression was examined on tissue sections of 32 SCCs of the head and neck. Generally, well-differentiated SCCs expressed E-cadherin, often as intensely as the normal stratified squamous epithelium (Fig. 3, a and b; Fig. 4 b, f, j, Table 1). A moderately differen-

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**Fig. 1.** E-cadherin expression in SCC cell lines of the head and neck by immunofluorescence. a and b, well-differentiated carcinoma cell line FaDu from the hypopharynx; c and d, moderately differentiated carcinoma cell line BA from the larynx; e and f, poorly differentiated carcinoma cell line WE from the larynx. a, c, and e, immunofluorescence staining; b, d, and f, corresponding phase contrast image. The well-differentiated carcinoma cell line expresses E-cadherin, whereas the moderately and poorly differentiated lines are negative. Bar, 30 μm.
E-cadherin expression in SCCs of Head and Neck Carcinomas

A summary of the clinical, histopathological, and E-cadherin expression data of the 32 SCCs with the data on the involved lymph nodes are shown in Table 1. The well-differentiated carcinomas generally expressed E-cadherin (12 cases were tested, two cases showed a heterogeneous pattern), and the moderately differentiated tumors exhibited intermediate E-cadherin staining or were heterogeneous (15 cases were tested), whereas the poorly differentiated carcinomas were all E-cadherin-negative (five cases were tested). In two cases we did not agree with the pathologists’ grading (tumors 11 and 27). In these, E-cadherin staining confirmed our own classification, e.g., tumor 27 was clearly poorly differentiated and E-cadherin-negative. Lymph node metastases were mostly found in cases where the primary tumor was E-cadherin-negative or heterogeneous. Remarkably, in seven of eight cases the infiltrated lymph nodes were E-cadherin-negative. These data suggest that lymph node metastasis in SCCs of the head and neck is largely incompatible with E-cadherin expression.

E-Cadherin Expression in SCCs of Head and Neck Analyzed by in Situ Hybridization. E-cadherin mRNA was identified on tissue sections of SCCs by in situ hybridization using a human E-cadherin antisense riboprobe (produced from the HC6-1 cDNA; see “Materials and Methods”). In the well-differentiated SCCs, E-cadherin expression was confined to islands of tumor cells in the connective tissue (Fig. 7, a and b). However, no E-cadherin mRNA was detected in all of the poorly differentiated tumors analyzed (Fig. 7, c and d). For comparison, high E-cadherin mRNA signals are shown in normal squamous epithelium of head and neck as well as in a neighboring well-differentiated SCC (Fig. 8a; Fig. 8b shows the control using the sense probe). E-cadherin mRNA and corresponding protein expression could be seen in enlargements of sections (Fig. 8, c–h).

**DISCUSSION**

In the present study we have examined the expression of the cell-cell adhesion molecule E-cadherin on tissue sections of human SCCs of the upper respiratory and digestive tract. *In vitro* experiments had shown previously that the absence or loss of function of E-cadherin leads to the disappearance of epithelial characteristics of the cells and generates higher invasiveness for extracellular matrices and embryonal heart tissue (21, 22). We show here that all poorly differentiated SCCs of the head and neck did not express E-cadherin, whereas the moderately differentiated tumors expressed E-cadherin in an intermediate or heterogeneous fashion. Well-differentiated SCCs expressed E-cadherin often as strongly as the normal stratified squamous epithelium. Remarkably, lymph node metastases were generally E-cadherin-negative, irrespective of whether they originated from poorly or moderately differentiated SCCs. These data suggest that E-cadherin plays a major role as a differentiation factor and invasion suppressor in epithelial tissues *in vivo*.

Several technical prerequisites made the present study feasible. Throughout we have obtained fresh human tumor material in close consultation with the surgeon (K. J.), which was clinically and histopathologically well characterized and was from untreated patients who had not undergone radio- or chemotherapy or previous surgical treatment. We then did careful presectioning of the material to first obtain a broad outline of the tumor structure, before we finally went into the detailed serial section analysis. We are also familiar with the properties of the anti-E-cadherin antibodies and the E-cadherin cDNA probe, which we have prepared and tested ourselves (22). Fur-
Fig. 3. E-cadherin expression in SCC tissues of the head and neck. a, normal stratified squamous epithelium from the hypopharynx; b, well-differentiated carcinoma from the larynx; c, moderately differentiated carcinoma from the hypopharynx; d, poorly differentiated carcinoma of the larynx. The well-differentiated carcinoma tissue shows a high expression of E-cadherin comparable with that of the normal epithelium, whereas the moderately differentiated carcinoma showed a weak signal. No E-cadherin expression is detectable in the poorly differentiated carcinoma. Tissues are from patients also listed in Table 1: a, case 1; b, case 2; c, case 22; d, case 31. Bar, 40 μm.
Fig. 4. Double immunofluorescence of E-cadherin and keratins in SCC tissues of the head and neck. Serial sections of SCCs were stained for E-cadherin (a-d) for cytokeratins (e-h), and with hematoxylin (i-l), a, e, and i, normal stratified epithelium of the pharynx; b, f, and j, well-differentiated SCC of the larynx; c, g, and k, moderately differentiated SCC of the hypopharynx; d, h, and l, poorly differentiated SCC of the hypopharynx. Note that the carcinoma tissues showed a gradual loss of E-cadherin with the loss of differentiation, but that the keratin staining was largely unchanged. The tumor in c, g, and k is heterogeneous; the central portion expressed E-cadherin and showed large cytoplasm, whereas the border area (arrows) was E-cadherin-negative and showed marginal cytoplasm. Tissues are from patients also listed in Table 1: a, case 12; b, case 5; c, case 2; d, case 30. Bar, 50 μm.
Table 1  Properties of the squamous cell carcinomas of head and neck

<table>
<thead>
<tr>
<th>Case</th>
<th>Age/sex/site</th>
<th>TNM</th>
<th>Differentiation grade</th>
<th>Primary tumor</th>
<th>Infiltrated lymph node</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>66/f/H</td>
<td>T2 NO MO</td>
<td>Well</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>69/m/L</td>
<td>T3 NO MO</td>
<td>Well</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>45/m/H</td>
<td>T3 NO MO</td>
<td>Well</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>43/f/H</td>
<td>T2 N1 MO</td>
<td>Well</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>64/m/L</td>
<td>T3 N1 MO</td>
<td>Well</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>57/m/L</td>
<td>T3 NO MO</td>
<td>Well</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>67/m/L</td>
<td>T3 NO MO</td>
<td>Well</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>52/m/H</td>
<td>T4 NO MO</td>
<td>Well</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>57/m/H</td>
<td>T4 NO MO</td>
<td>Well</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>61/f/O</td>
<td>T4 NO MO</td>
<td>Well</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>64/m/H</td>
<td>T1 N3 MO</td>
<td>Poorly</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>77/m/F</td>
<td>T2 N1 MO</td>
<td>Well*</td>
<td>+/-</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>39/f/O</td>
<td>T4 NO MO</td>
<td>Moderately</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>51/f/O</td>
<td>T2 N1 MO</td>
<td>Moderately</td>
<td>++/+-</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>50/m/L</td>
<td>T5</td>
<td>Moderately</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>60/f/O</td>
<td>T2 NO MO</td>
<td>Moderately</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>57/m/L</td>
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<td>Moderately</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>61/m/O</td>
<td>T3 NO MO</td>
<td>Moderately</td>
<td>+/-</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>55/f/O</td>
<td>T4 NO MO</td>
<td>Moderately</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>51/f/L</td>
<td>T2 N1 MO</td>
<td>Moderately</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>62/m/H</td>
<td>T2 N1 MO</td>
<td>Moderately</td>
<td>+/-</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>62/m/H</td>
<td>T2 N2MO</td>
<td>Moderately</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>66/f/F</td>
<td>T3 N2MO</td>
<td>Moderately</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>72/m/O</td>
<td>T2 N2MO</td>
<td>Moderately</td>
<td>+/-</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>47/m/F</td>
<td>T4 N2MO</td>
<td>Moderately</td>
<td>+/-</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>57/m/O</td>
<td>T3 N3 MO</td>
<td>Moderately</td>
<td>+/-</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>54/f/L</td>
<td>T4 N2MO</td>
<td>Moderately</td>
<td>+/-</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>62/f/O</td>
<td>T2 NO MO</td>
<td>Poorly</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>65/m/H</td>
<td>T3 N1 MO</td>
<td>Poorly</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>47/m/H</td>
<td>T2 N2MO</td>
<td>Poorly</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>49/f/L</td>
<td>T4 N1 MO</td>
<td>Poorly</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>50/m/H</td>
<td>T3 N3 MO</td>
<td>Poorly</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

* Site of tumor: F, floor of mouth; O, oropharynx; H, hypopharynx; L, larynx.
* TNM staging (25) as performed by the Department of Otorhinolaryngology, Medical School, University of Essen. T, tumor size; N, lymph node infiltration; M, distant metastasis. N0, no infiltration; N1, one infiltrated lymph node <3 cm; N2a, several infiltrated lymph nodes <6 cm on one side; N2c, same, but on both sides; N3, all infiltrated lymph nodes >6 cm; MO, no distant metastasis; Tis, carcinoma in situ.
* Tumor grading as determined by the Department of Pathology, Medical School, University of Essen. "Well" is defined as moderately differentiated. * The area of this tumor we have analyzed was clearly poorly differentiated.

E-CADHERIN EXPRESSION IN HEAD AND NECK CARCINOMAS

Invasion of the infiltrated lymph nodes of SCCs showed that they were generally E-cadherin-negative. The results suggest that preferentially E-cadherin-negative carcinoma cells migrate from the primary tumors and colonize the neighboring lymph nodes. Alternatively, local lymphatic vessels might have better access to E-cadherin-negative tumor tissue, and by this E-cadherin-deficient cells might be preferentially mobilized. The one exception (the lymph nodes of tumor 23 in Table 1) could be due to other factors (e.g., motility factors) which can mobilize carcinoma cells without affecting E-cadherin expression (26, 27). The underlying molecular mechanism for E-cadherin down-regulation in the SCCs is not known. It could be due either to mutations in the E-cadherin structural gene or to indirect suppression of E-cadherin gene expression. Concerning the first possibility, a functionally inactive E-cadherin was recently created by the introduction of a point mutation into the cDNA coding for the extracellular domain (28). Furthermore, a new tumor suppressor gene has recently been identified at the locus on chromosome 16q, bands 22.1–23.2, which is affected in most of the dedifferentiated liver carcinomas (29) as well as in breast and prostate carcinomas (30, 31). E-cadherin is a good candidate for this impossible invasion suppressor gene, since it is located on chromosome 16q, band 21.1 (32). Second, epithelial-specific regulatory elements have recently been identified in the E-cadherin gene promoter, which are active in certain differentiated but not dedifferentiated breast carcinoma cell lines (33). It is not yet known whether 16q mutations in fact exist in head and neck tumors or whether the postulated epithelial specific promoter elements play a role. Recently, various other components have been described which might be involved in invasion and metastasis (deleted in colon carcinoma, carcinoembryonic antigen, CD44, and nm 23; cf. Refs. 34–37). Deleted in colon carcinoma, carcinoembryonic antigen, and CD44 also seem to influence the adhesive properties of the tumor cells.

E-cadherin expression was also studied recently in other types of carcinomas. A highly undifferentiated hepatocellular carci-
Fig. 6. E-cadherin expression by immunofluorescence in infiltrated lymph nodes of SCCs of the head and neck. a, c, and e, E-cadherin expression; b, d, and f, corresponding staining of cytokeratins. The lymph node in a and b is infiltrated by small groups of E-cadherin-negative carcinoma cells (the cytokeratin-positive cells are marked by arrows in b), and the lymph node in c and d is virtually overgrown by E-cadherin-negative carcinoma cells. e and f, the only case with E-cadherin-positive lymph nodes. Lymph nodes are from cases 12 (a and b), 27 (c and d), and 23 (e and f) (see Table 1). Bar, 40 μm.
Fig. 7. E-cadherin expression as analyzed by in situ hybridization of SCC tissues of the head and neck. a and b, well-differentiated SCC; c and d, poorly differentiated SCC. Tissues were from patients listed in Table 1: a, case 5; c, case 31. Sections were hybridized with E-cadherin antisense riboprobe as described in “Materials and Methods.” After autoradiography, sections were counterstained with hematoxylin to reveal the location of cell nuclei and epithelial structures. The sections were then analyzed by darkfield (a and c) and brightfield (b and d) illumination. E-cadherin mRNA is strongly expressed in the tumor islands of the well-differentiated and not expressed in the poorly differentiated carcinoma. ——, borderline between carcinoma (ca) and connective tissue (ct). Bar, 40 μm.
Fig. 8. In situ hybridization of E-cadherin in normal epithelium and carcinoma tissue of the head and neck. Serial sections of both normal epithelium (ep) and a neighboring well-differentiated SCC (ca) separated by connective tissue (ct) were hybridized with E-cadherin antisense (a) and sense (b) riboprobes. c–h, enlargements of characteristic regions of the normal epithelium (* in a) and the SCC (x in a); c and f, staining with the antisense riboprobe; d and g, immunofluorescence staining with antibody 6F9; e and h, staining with hematoxylin. Tissue is from patient 3 in Table 1. Bars: b, 530 μm; c, 45 μm; f, 90 μm.


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