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ARTICLE

Changes of Cell Adhesion and Extracellular Matrix (ECM) Components in Cervical Intraepithelial Neoplasia

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Cell-cell and cell-extracellular matrix interaction is crucial in tumor progression. Tight junction (TJ) proteins as occludin and claudins (CLDNs) play important role in this process together with several extracellular matrix components, as syndecan. Our previous work suggested significant changes in the expression of claudins even in the early stages of cervical carcinogenesis. The aim of our present work was to study the expression of occludin and syndecan-1, as compared to CLDNs, in early phases of cervical carcinogenesis. Paraffin sections of 50 samples were studied by immunohistochemistry, including cervical intraepithelial neoplasias (CIN-I-II-III), in situ carcinomas (CIS) and normal cervical samples. Occludin and CLDN-2 were found colocalized in the basal layer, while syndecan-1 and CLDN-1, -4 and -7 were coexpressed in the parabasal and intermedier layers in normal epithelia. Intensity of occludin staining decreased in CIN/CIS lesions, although it was more extended towards the upper epithelial layers with inverse relation with grades, as seen in the case of CLDN-2 expression. CLDN-1, -2, -4 and -7 were detected in the entire epithelium in CIN, showing decrease in CIS. The progression of CIN was associated with reduced syndecan-1 expression, in contrast to CLDN-1, -4 and -7 which increased toward CIS. The obtained data suggest that significant changes occur in the composition of cell adhesion complexes even in early stages of cervical carcinogenesis. The pattern of expression is characteristic for the alteration, the changes in the different components, however, are not parallel with each other. (Pathology Oncology Research Vol 11, No 1, 26–31)

Key words: occludin, claudin, cervical intraepithelial neoplasia, syndecan-1, carcinogenesis

Introduction

During recent years, attention was drawn to the role of cell adhesion in tumor development and progression.[10,11] Cell-cell and cell-extracellular matrix interaction is crucial with regard to tumorous transformation and tumor spreading.[24] There are numerous data indicating that the expression of syndecan – an important transmembrane proteoglycan – undergoes changes during the development of cervical carcinomas.[22,24,28] Syndecan-1 is expressed in the normal cervical epithelium, while being significantly less prevalent in invasive carcinomas.[14,22] Recent data have verified the role of cell surface heparan sulfate proteoglycans (HSPG) in human papilloma virus (HPV) infection, functioning as the cellular receptor for the virus.[9,30] From these HSPGs, syndecan-1 is among the most important components, possibly serving as the primary receptor for HPV.[30] Several data have revealed that detachment of the extracellular domain of syndecan and its enhanced presence in the extracellular matrix (ECM) can be regarded as an unfavorable prognostic marker in case of many tumors.[32,38,40]

In respect to cell junctions, another molecule family – the claudins (CLDNs) – has also received special attention throughout the past years.[37] First described in 1998, this family currently counts 24 members according to our pre-

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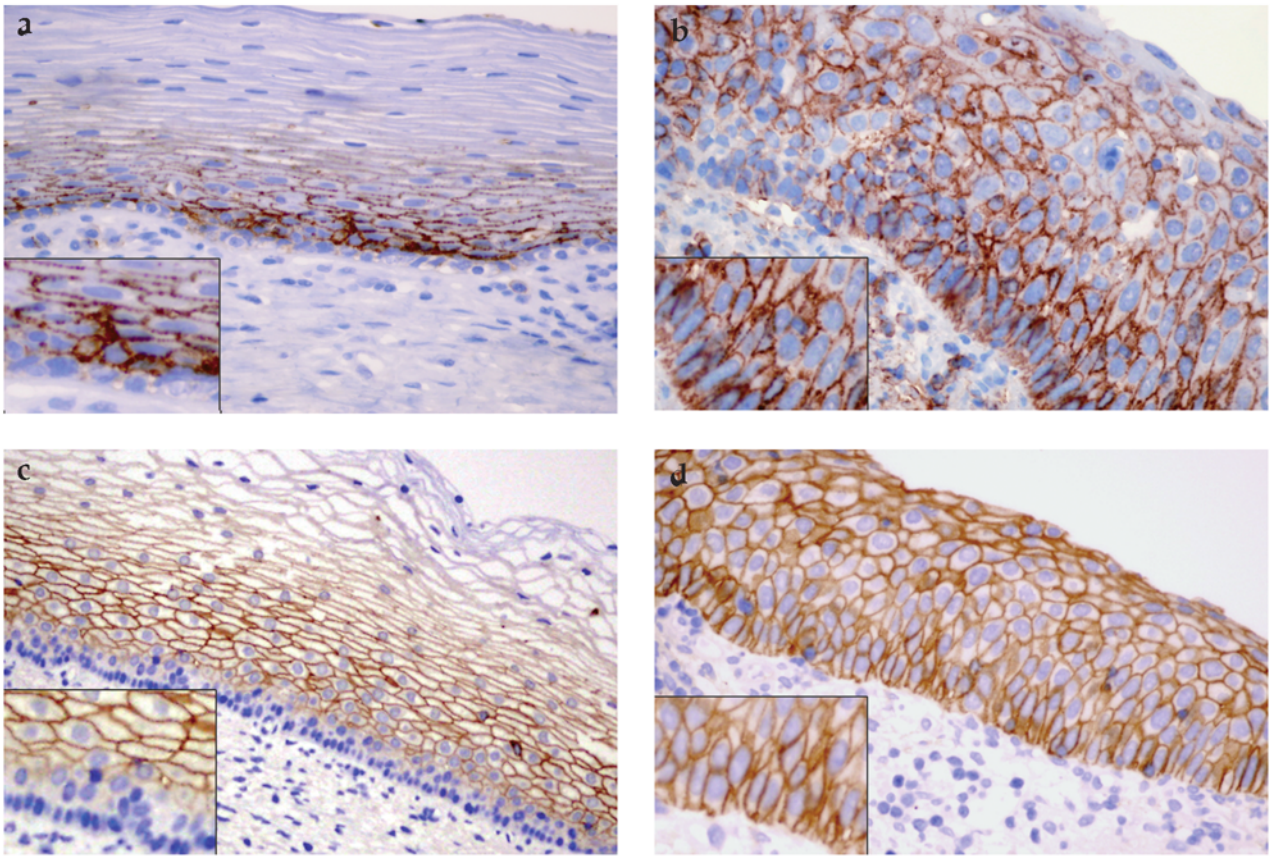

Figure 1. *Immunohistochemical detection of syndecan-1* ***(a, b)*** *and claudin-1* ***(c, d).*** *In normal cervical epithelium* ***(a, c)*** *the membranous positivity is located in the suprabasal layers. Note the extended reaction in CIN-II lesions* ***(b, d)*** *(x200).*

sent knowledge.[6,36] CLDNs play a role in the structural establishment of tight junctions (TJs), with special role in determining epithelial cell polarity and intercellular permeability.[6,10,17] Data are also available revealing the importance of changes in expression – decrease or increase – of certain claudins in the development of several tumors, as colorectal carcinomas,[20] tumors of the pancreas,[23] breast,[16,35] prostate[41] and ovaries.[27] Recently, our group studied the expression of different CLDNs in premalignant and malignant cervical lesions. It has been shown that following an initial increase, a significant decrease is detectable during progression.[31] Other earlier identified components of TJs, as occludin, junctional adhesion molecule (JAM) Z01, Z02, etc. are also thought to be principal molecules that contribute to cell polarity and barrier function.[8,15,26] Abnormalities of these components may therefore result in structural alterations in different neoplasias as well.[1,5,21,29,33,34]

The members of these molecule groups have similar roles at least functionally, and are important regarding both extracellular matrix-cell communication and signal transfer, as extra- and intracellular domain-bearing transmembrane proteins. Prompted by our previous observations on CLDNs,[31] the aim of the present work was to compare the changes in other TJ proteins, like occludin, with correlation to each other and to extracellular matrix components, such as syndecan-1.

Materials and methods

Our studies were performed using archival samples collected for diagnostic purposes, with permission obtained from the Regional Ethical Committee of Budapest, Hungary (#172/2003). A total of 50 samples were analyzed, including 10 normal cervical epithelia, 10 CIN-I, 10 CIN-II, 10 CIN-III lesions, and 10 in situ carcinomas (CIS).

The tissue samples were routinely embedded in paraffin following formalin fixation. Three to four μm thick sections were stained with hematoxylin and eosin (HE). Details of the tissue preparation and controls used are described elsewhere.[25,31]

Immunohistochemical reaction

Sections were deparaffinized routinely and subjected to immunohistochemistry. Antibodies to syndecan-1 and claudin-1, -2, -3, -4 and -7 were processed by Ventana ES

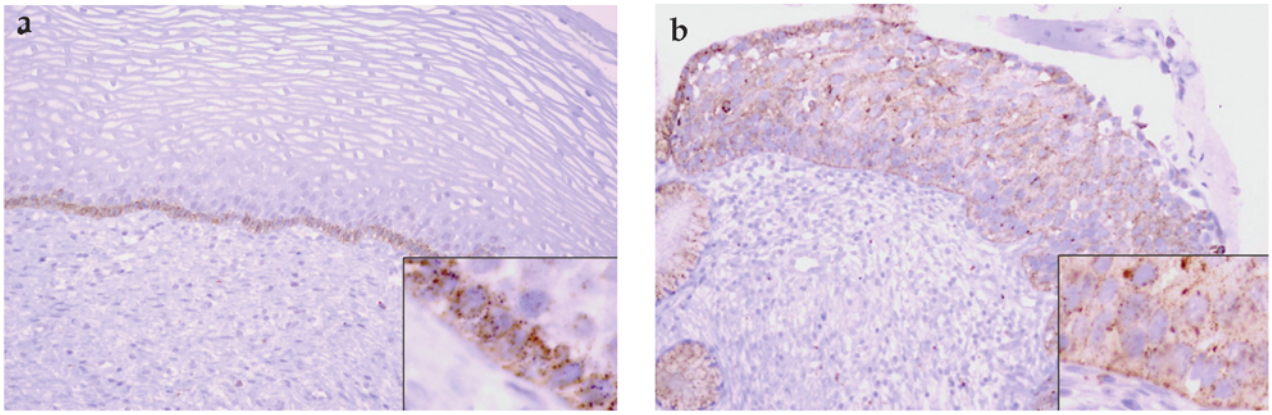

Figure 2. *Claudin-2* ***(a, b)*** *and occludin* ***(c, d)*** *immunoreaction. Positivity is located in the basal layers in normal cervical epithelia* ***(a, c).*** *CIN-II: the granular membranous positivity for claudin-2 involves the upper epithelial layer* ***(b),*** *irregular, extended reaction for occludin* ***(d)*** *(x200, insert: x400).*

automatic immunostainer (Ventana Medical Systems Inc., Tucson, Arizona), while occludin antibody was processed manually.

Human anti-syndecan-1 mouse monoclonal antibody (Serotec Ltd., Oxford, UK) at a dilution of 1:100, claudin-2 (Zymed 18-7363, San Francisco, CA), claudin-4 (Zymed 18-7341) mouse monoclonal and claudin-1 (Zymed 51-9000), claudin-3 (Zymed 34-1700), claudin-7 (Zymed 34-9100) rabbit polyclonal antibodies at 1:100 dilution in PBS were applied overnight at 4°C, then incubated with peroxidase-labeled secondary antibodies (Ventana 2532189) and diaminobenzidine (DAB) (Ventana 2532190) as chromogen. Target retrieval was performed with retrieval solution (Dako S1699) before the application of primary antibodies. Occludin was detected using rabbit polyclonal antibody (Zymed 71-1500) at 1:300 dilution in PBS, overnight at 4°C, followed by Envision™ visualization system (Dako K4003) and processed with DAB (Ventana). Proteinase K (Dako S3020), (ready to use) digestion for 15 minutes was used for antigen retrieval before primary antibody.

Tissues were blocked for endogenous peroxidase activity with 3% H_2O_2. Negative controls for nonspecific reaction were processed and revealed no signal. The immune reactions were evaluated by two independent observers. Localization of immunoreaction in the different cell layers was divided as follows: basal, parabasal (lower third excluding the basal layer), intermediate, superficial (upper one third).

Results

Syndecan-1 expression in the normal cervical epithelia showed rather intensive, continuous membranous staining at the cell surface. The reaction was not detected in the basal and superficial layers, the cells in the parabasal and intermediate layers, however, were strongly positive (*Figure 1a*). In the CIN-I-II-III and CIS lesions, syndecan-1 expression displayed an intensity similar to that observed in the positive layers of the normal epithelia. The reaction was delineating the cell borders, similar to the appearance noted in normal epithelia, though being more extended (*Figure 1b*). Positivity included the basal layer and upper half of the dysplastic epithelia. The distribution of positive cells, however, was less even, smaller or larger negative foci were seen in more advanced CIN lesions (*Figure 1b*).

Claudin-1, -4 and -7 gave similar membranous reaction as syndecan-1, though the intensity was somewhat lower in the case of claudin-7, and even weaker for CLDN-4 (not shown). In the normal epithelia the reaction did not extend to the basal layer, while being positive in the parabasal and intermediate layers (*Figure 1c*). The superficial epithelial layer was found to be negative. The reaction was more extended in the CIN-I lesion than in the normal epithelia, involving the whole epithelia with the exception of a quarter of the upper epithelial layer, but including the basal layer. In case of the CIN-II-III (*Figure 1d*) and CIS lesions, claudin-1 (*Figure 1d*) and claudin-4 and -7 were notable in all epithelial layers and all cells reacted with the antibodies.

Claudin-2 was localized in the basal layer of the normal epithelia, while showing lower intensity reaction in the parabasal layer and no reaction at all in the intermediate and superficial layers (*Figure 2a*). Contrary to the other claudins, the reaction was found to be granular-like and discontinuous on the cell surface (*Figure 2a,* insert). Regarding CIN-I-III and CIS lesions, claudin expression was rather enhanced, appearing in every epithelial layer (*Figure 2b*).

Claudin-3 proved to be negative in both the normal and abnormal squamous epithelia, and was completely negative in all other layers.

Occludin gave strong membranous reaction in the basal cell layer while no positivity was detected in the upper layers (*Figure 2c*). Decrease in the intensity of reaction was noted in CIN lesions. Conversely, however, more cells were expressing occludin as compared with normal epithelia. Positive membranous staining of various intensity was notable in all layers in the CIN I-II lesions (*Figure 2d, 3a*). Interestingly, however, the intensive staining almost completely disappeared in the CIS lesions and the extended areas were completely negative (*Figure 3b*).

Discussion

Cell-cell relationships have a decisive role in both tumor formation and cell-to-cell communication.[10,11] The expression of the molecules involved may either become enhanced or diminished during the course of carcinogenesis.

In our study occludin and claudin-2 were found coexpressed in the basal cell layer, syndecan-1 and the other claudins in the upper layers of the normal cervical epithelium, which suggests a possible functional and structural connection between the molecules involved in the same localization. Interestingly, however, all proteins studied showed a markedly extended increase in the early CIN lesions. This implies a significant rearrangement of cell junctions and ECM transmembrane proteins even in the early phase of cervical carcinogenesis. During tumorous transformation, however, syndecan-1 and occludin exhibit gradual decrease, while the expressions of CLDN-1, -2, -4 and -7 show definite enhancement at least in the CIN/CIS lesions, with no decrease at all. This indicates that in the early phase of tumor formation the changes in the components of the extracellular matrix and cell adhesion are not necessarily parallel.

Our earlier study demonstrated enhanced expression of CLDN-1, -2, -4 and -7 in the CIN and CIS lesions and in invasive squamous carcinomas as compared with normal cervical squamous epithelia.[31] The increased expression of CLDNs has been detected by molecular biological methods in several tumors types as colorectal carcinomas,[20] tumors of the pancreas,[23] breasts,[16,35] prostate[41] and ovaries.[27] These studies primarily dealt with progressive invasive tumors, thus our own observations are of particular interest since they involved studies on the early stages of carcinogenesis, and point to the differences in expression of certain TJ and ECM proteins. Marked increase of extended CLDN-1, -2, -4 and -7 expressions was demonstrated in the CIN and CIS lesions, without decrease in the highest grade. Syndecan-1,[14] occludin[33] and CLDN expression[31] has been noted to decrease in invasive carcinomas. It is interesting, however, that all the adhesion proteins in the present study showed strong expression in the early stage of neoplastic transformation. This might suggest tighter cell-to-cell relationships prior to invasion, though it could be that this increase in expression indicates a shift in ratios, the abnormal architectural restructuring of cell junctions, which might not necessarily mean functional increase of cell adhesion. The importance and role of the decrease, that is, looser cell adhesion throughout tumor progression is apparent, but the significance of the enhanced expression of these molecules noted in the early stages still needs to be clarified.

The strikingly high expression of CLDN-4 in carcinomas of the pancreas[23] and ovaries[27] is particularly noteworthy. CLDN-4 is known to be the enterotoxin receptor of Clostridium perfringens, which could have therapeutic consequences, though the pathophysiological interpretation of the enhanced expression of this molecule is still to come. It is known that in the *in vitro* culture of pancreas carcinoma, where CLDN-4 is highly expressed, Clostridium perfringens toxin treatment kills the tumor cells.[23] The observed high expression levels of occludin and CLDN-1, -2, -4 and -7 in the CIN and CIS lesions in our study could also support their use as therapeutic targets in the future, even in stages preceding invasion.

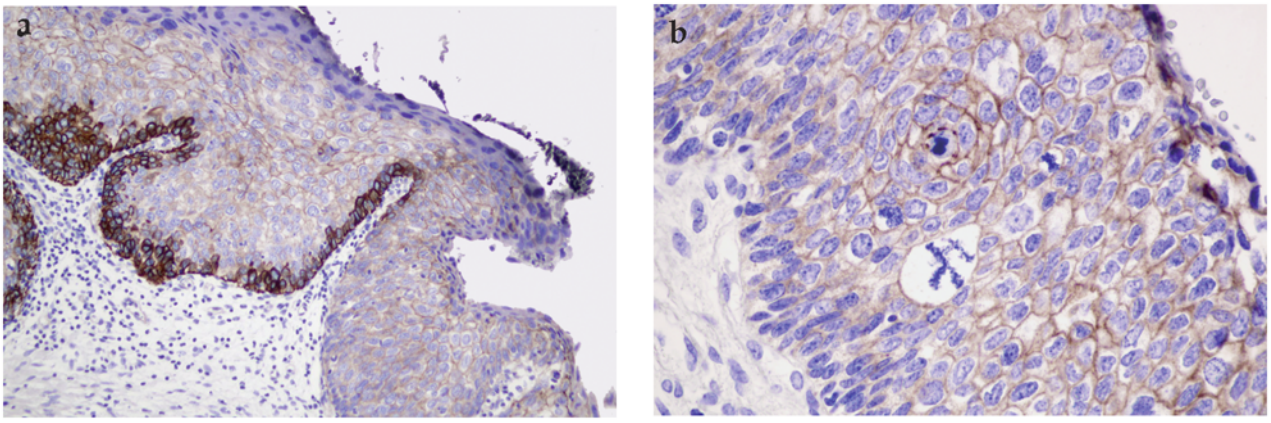

Figure 3. *Occludin immunohistochemistry. Positivity is still seen in CIN-II* ***(a),*** *significant decrease or loss in CIN-III* ***(a, left half)*** *and CIS* ***(b)*** *(x200, x400).*

In our work, after the initial increase in CIN-I lesions, the expression of syndecan-1 exhibited a steady decrease until reaching the stage of invasive cancer, which could most probably be explained by the detachment of the extracellular domain of this transmembrane protein.[40] Conversely, the increased expression of CLDN-1, -2, -4 and -7 in CIN lesions is indicative of enhanced cell adhesion – a phenomenon followed by the declining expression of these molecules in the later, invasive phase.[31] The expression of other cell adhesion molecules, as Ep-CAM, displays similar enhancement in actively proliferating cell populations, thus in CIN/CIS lesions, since the cells do not enter the phase of terminal differentiation.[18] The appearance of CLDN-1, 4-, and -7 in the basal layer in CIN lesions may also indicate their role in the early phase of cervical carcinogenesis, similarly to that of Ep-CAM molecules.[18] All these denote a disturbance in cell proliferation and differentiation. The manifestation of CLDN-1, -4, and -7 and syndecan-1 at the basal cell layer in CIN/CIS lesions may be an important marker of the interruption of normal differentiation.

There are several data to indicate that in case of syndecan the extracellular or transmembrane localization of the molecule and its detachment from the cell surface are factors decisive in the development of certain tumors.[2,3,4] This corresponds to earlier observations according to which the decrease of syndecan and occludin expression on the cell surface can be considered as the sign of tumor progression,[7,19] the decrease being more pronounced in invasive carcinomas.[33]

Changes of cell junctions and cell polarity have an essential role in tumor formation.[21,26] These processes are influenced by TJ molecules as occludin and CLDNs, partly by changing the „barrier" function, partly by affecting the *wnt*-signal transduction system. There are similar observations in connection with other cell adhesion molecules too, as in case of Ep-CAM[18] and integrins.[12,13]

All these changes might be related to one of the most important etiological factors of cervical carcinoma, HPV. Earlier on, reference was made to the role of HSPGs – and within these syndecan-1 – as HPV receptors.[9,30] Furthermore, data are available on the upregulation of certain cell adhesion molecules, as Ep-CAM, due to the effect of HPV infection.[39] Currently, no reference is available regarding a possible connection between the high expression of occludin and CLDNs and HPV infection, though based on our results, such a connection cannot be excluded and should call for further research on the subject.

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