Original Articles

Down regulation of estrogen receptor expression is an early event in human papillomavirus infected cervical dysplasia

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Summary

Purpose: To study the alterations in hormonal sensitivity in relation to proliferative activity during the development of cervical dysplasia in women infected with high-risk human papillomavirus (hr-HPV).

Methods: Three to five biopsies of the cervix of eight patients were taken at colposcopy. Dysplasia was detected in 22 of the 32 biopsies, and 20 of these 22 biopsies contained hr-HPV. The labeling index (LI) as well as the intensity of staining of the MIB-1-, estrogen receptor (ER)-, and progesterone receptor (PR)- expression was assessed in each biopsy, including normal epithelium directly adjacent to the dysplastic lesions.

Results: Statistical analysis showed a significant increase in the MIB-1 LI with increasing severity of the dysplasia. The ER LI and ER intensity of staining in dysplastic lesions, as well as in morphologically normal epithelium directly adjacent to the dysplasia, showed a significant inverse relation with the severity of the dysplasia. The PR LI and intensity of staining did not differ between normal epithelium and dysplasia. The ER/MIB-1 ratio (including the ER LI and ER intensity of staining), and the PR/MIB-1 ratio (intensity of staining only) in dysplastic lesions showed a significant inverse relation with the severity of the dysplasia, while no alterations in these ratios were observed in morphologically normal epithelium adjacent to the dysplasia.

Conclusion: Down regulation of ER expression may be the first alteration to take place in normal epithelium during the development of cervical dysplasia in women infected with hr-HPV. The significant decrease in the ER/MIB-1-, and PR/MIB-1 –ratio in progressively dysplastic lesions indicates a loss of normal growth control by sex steroid hormones, which is not observed in normal epithelium.

Key words: Estrogen receptor; Progesterone receptor; Hyperproliferation; Cervical dysplasia.

Introduction

Cancer of the uterine cervix ranks number two worldwide in cancers of women, accounting for 6% of all malignancies. Epidemiological, clinicopathological, and molecular biological studies over the past two decades have convincingly demonstrated that infections with high-risk human papillomavirus (hr-HPV) are etiologically related to the development of pre-malignant cervical lesions and invasive cervical cancer [1-7]. Hr-HPV infections cause expression of oncoproteins, of which especially E6 and E7 disturb cell-cycle regulators by inactivating p53, and pRb, respectively. These disturbances result in hyperproliferation of the epithelium [8, 9]. Hyperproliferation shows a strong correlation with the degree of cervical dysplasia and can be visualized with the monoclonal antibody MIB-1. MIB-1 recognizes the Ki-67 antigen, an antigen that is expressed in all proliferating cells [9-11].

The long-term use of oral contraceptives, as well as exogenous estrogen supplementation have been identified as independent risk factors for the development of HPV-mediated cervical cancer [6, 12, 13]. A relation between the degree of dysplasia and estrogen receptor (ER), or progesterone receptor (PR) expression has been described previously, but the results of several studies seem to contradict each other [8, 14-19].

In the present study, the proliferative activity in relation with ER and PR expression was investigated in normal and dysplastic lesions within the uterine cervix of an individual patient, as well as among different patients, in order to study alterations in hormonal sensitivity in relation to proliferative activity during the development of cervical dysplasia in women infected with hr-HPV.

Material and Methods

Patients

Eight patients referred to the colposcopy clinic of the Radboud University Nijmegen Medical Center with an abnormal cervical scrape were included in this study. The mean age was 37 years (range 32-46 years). None of the patients had previously been treated for dysplasia of the uterine cervix. Colposcopy was performed in the luteal phase of the menstrual cycle in five patients, during the second half of an oral contraceptive cycle (patient 7), and during daily intake of a progestative (patient 5). In one patient the phase of the menstrual cycle at colposcopy was not registered (patient 8).

The cervix was assessed and mapped at colposcopy using acetic acid. After application of a local anesthetic, three to five biopsies were taken from the uterine cervix of each patient using a small diathermy loop. All cervical areas with a colposcopically suspected different degree of dysplasia were biopsied, including an area of colposcopically normal epithelium.

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Subsequently, the transformation zone was removed by large diathermy loop excision in order to complete treatment of all cervical abnormalities. In total 32 separate biopsies of the eight patients were taken and the localization of each biopsy was marked in the patient's record.

All biopsies were processed separately and embedded in paraffin to avoid cross-contamination with HPV. Histopathological examination was done on sections of each biopsy. Dysplasia was detected in 22, mild dysplasia (MiD) in four, endocervical atypia in one, moderate dysplasia (MoD) in three, severe dysplasia (SD) in nine, carcinoma in situ (CIS) in two, and micro-invasive carcinoma (MIC) in three biopsies. In the ten biopsies without dysplasia, three showed squamous metaplasia, six showed normal squamous epithelium, and one showed normal endocervical epithelium (Table 1).

Subsequent sections of the 30 biopsies that contained squamous epithelium were tested for the presence of hr-HPV, and assessment of MIB-1-, ER-, and PR- expression.

High-risk HPV detection

A 3-μm-thick section of each biopsy was processed for HPV detection with a broad-spectrum short polymerase chain reaction fragment (SPF_{10} HPV-PCR) [21]. In case of a positive result, subsequent HPV genotyping was done in a reverse hybridization line probe assay (LiPA), which identifies 25 different HPV genotypes simultaneously. HPV genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, and 68 were regarded as high risk, and HPV genotypes 6, 11, 34, 40, 42, 43, 44, 54, 70, and 74 were regarded as low risk. This detection method was validated before and was found to be highly sensitive, specific, and reproducible, also in cases with multiple HPV infections [21-25].

Immunohistochemistry

Three-μm-thick paraffin sections were mounted onto polylysine-coated slides and dried overnight at 37°C. Paraffin sections were dewaxed in xylene and rehydrated in a standard series of graded alcohols. Rehydrated slides were placed in a citrate buffer (10mM, pH 6.0) and heated in a household microwave oven at 90°C for 20 min. After microwave preprocessing, the sections were allowed to cool down to room temperature. Subsequently, the slides were washed in phosphate-buffered saline (PBS, pH 7.4) [9].

An indirect immunoperoxidase technique was used to visualize the Ki-67 antigen. The sections were incubated with the mouse monoclonal antibody MIB-1 (Immunotech S.A., France) 1:40 in PBS with 2% normal calf serum overnight at 4°C and subsequently incubated with a rabbit anti-mouse peroxidase (Dako, Denmark) 1:100 in PBS for 60 minutes at room temperature. The peroxidase-labeled complex was developed with diaminobenzidine (DAB; Vector Laboratories) for 4 min at room temperature and intensified with 5% CuSO4 for 5 min at room temperature. All incubation steps were followed by three washes in PBS of 5 min. Subsequently the slides were slightly counterstained with Mayer's hematoxylin, dehydrated in ethanol and xylene, and finally mounted.

In order to detect ER and PR expression, subsequent sections of the biopsy were pretreated as for MIB-1, followed by incubation with normal horse serum for 10 min at room temperature. The sections were incubated for 60 min at room temperature with mouse IgG serum (Dako, Denmark) 1:200 in PBS for ER, and 1:900 in PBS for PR, followed by incubation with a biotine-labeled, horse anti-mouse serum (Dako, Denmark) 1:200 for 30 min at room temperature. The peroxidase-labeled complex was developed as for MIB-1.

Analysis

The labeling index (LI), as well as the intensity of the staining of the MIB-1-, ER-, and PR- expression were assessed in each dysplastic squamous lesion, and in normal epithelium. Additionally, in 18 of the 20 sections that contained dysplasia with areas of normal squamous epithelium, the MIB-1-, ER-, and PR- expression were also assessed in the normal epithelium directly adjacent to the dysplasia. The intensity of the staining (no staining = 0%, slight = 33%, moderate = 67%, severe = 100%) and the percentage of nuclei that were stained (LI) were scored for the full thickness of the epithelium. The relation between the proliferative activity and ER-, and PR- expression was studied by assessing the ratios of the ER LI, ER intensity of staining, PR LI, and PR intensity of staining with the MIB-1 LI in the different degrees of dysplasia.

Statistical analysis

Statistical analysis consisted of Jonckheere-Terpstra's distribution-free test for ordered alternatives and unpaired t-tests for independent variables, in order to detect significant relations of the MIB-1-, ER-, PR-expression, and the ratios with the degree of dysplasia.

Results

High-risk HPV detection

In Table 1 it can be seen that hr-HPV was detected in all biopsies in which dysplasia was observed at histopathological examination, except for a MiD lesion in patients 4 and 5. Hr-HPV was detected in two of the three biopsies with squamous metaplasia, in two of the six biopsies with normal squamous epithelium, and in one patient with normal endocervical epithelium. In all patients with SD as the most severe lesion (patients 1, 3, 5, and 6), more than one genotype of hr-HPV was detected within the cervix, and even within a single biopsy (Table 1). In the other four patients a single hr-HPV genotype was detected. Two of these patients had MIC, and one patient had a CIS. In the patients with MIC, HPV 16 was detected throughout the cervix, and even the SD lesions in these patients contained only this single genotype of HPV.

MIB-1 expression

The Jonckheere-Terpstra's test for ordered alternatives showed a highly significant ($p < 0.001$) increase of the mean MIB-1 LI, and no relation of the MIB-1 intensity of the staining ($p = 0.29$) with the severity of the dysplasia in the biopsy (Figure 1a). The MIB-1 LI was $\geq 33\%$ in all biopsies with SD, CIS, and MIC, and $< 33\%$ in all biopsies without dysplasia (Table 1). All areas of normal epithelium adjacent to areas of dysplasia had a MIB-1 LI $\leq 33\%$. There was no significant relation between the MIB-1 LI ($p = 0.19$) or MIB-1 intensity ($p = 0.28$) in normal epithelium adjacent to dysplastic epithelium with increasing severity of that dysplasia (Figure 2a). There was no significant relation between MIB-1 LI and the presence of more severe dysplastic lesions in the same cervix, the presence of HPV 16, other HPV genotypes, or multiple hr-HPV infections.

Table 1. — *Detected HPV genotypes, histological grading, LI and intensity of staining (Int.) for MIB-1-, ER-, and PR- expression of each biopsy.*

Pat.	Biopsy	HPV	Histological grading	MIB-1%		ER %		PR %	
				LI	Int.	LI	Int.	LI	Int.
1	1	Neg	Sq metaplasia	25	67	33	100	25	67
	2	Neg	Sq metaplasia	42	67	42	100	25	33
	3	58	SD	58	67	25	33	17	33
	4	33/58	MoD	17	67	33	100	8	33
2	1	16	CIS	75	100	0	0	0	0
	2	16	MoD	17	67	25	67	17	100
	3	Neg	Nl sq epith	8	67	33	100	8	33
	4	16	Nl endocx epith	–	–	–	–	–	–
3	1	33	SD	33	100	42	67	17	33
	2	31/33/52/66	SD	33	100	17	33	17	33
	3	33/66	Nl sq epith/inflam	25	100	25	100	17	33
	4	66	Endocx atypia	–	–	–	–	–	–
4	1	51	MoD	25	100	17	33	0	0
	2	Neg	MiD	33	100	17	67	25	67
	3	Neg	Nl sq epith	25	67	50	100	17	67
5	1	16/31	SD	42	100	0	0	0	0
	2	Neg	MiD	8	100	25	67	8	33
	3	Neg	Nl sq epith	8	67	17	67	0	0
	4	16	Sq metaplasia	17	67	25	67	0	0
6	1	16/18	SD	75	100	0	0	0	0
	2	16/18/52	SD + Endocx atypia	50	100	0	0	0	0
	3	16/18	SD	42	100	0	0	0	0
	4	16	MiD	42	33	17	67	0	0
7	1	16	CIS	75	67	0	0	50	33
	2	16	MiD	17	67	25	67	17	33
	3	16	Nl sq epith	17	67	25	67	17	33
	4	16	MIC	75	67	0	0	75	33
8	1	16	SD	75	67	17	33	17	33
	2	16	SD	67	67	17	33	25	33
	3	16	Nl sq epith	17	67	25	67	0	0
	4	16	MIC	92	67	0	0	0	0
	5	16	MIC	75	67	0	0	0	0

Nl sq epith = Normal squamous epithelium, inflam = inflammation; Nl endocx epith = normal endocervical epithelium; MiD = mild dysplasia; MoD = moderate dysplasia; SD = severe dysplasia; CIS = carcinoma in situ; MIC = microinvasive carcinoma; ER = estrogen receptor; PR = progesterone receptor.

ER expression

The Jonckheere-Terpstra's test for ordered alternatives showed a highly significant decrease of ER LI ($p < 0.001$), and of ER intensity ($p < 0.001$) with increasing severity of the dysplasia in the biopsy (Figure 1b). ER expression was 0 in all biopsies with MIC or CIS, and in four of the nine biopsies with SD. All biopsies with MoD or less showed ER expression (Table 1). All areas of normal epithelium adjacent to dysplastic lesions, except for one area next to MIC (patient 7), showed a positive ER expression. There was a significant decrease in ER LI ($p = 0.05$) and in the ER intensity of staining ($p = 0.03$) in normal epithelium adjacent to dysplasia with increasing severity of that adjacent dysplasia (Figure 2b). There was no difference in the ER LI or intensity of staining regarding the presence of more severe dysplastic lesions in other parts of the cervix, the presence of HPV 16, other HPV genotypes, or multiple hr-HPV infections.

PR expression

The Jonckheere-Terpstra's test for ordered alternatives did not show a significant relation of the PR LI ($p = 0.16$) and PR intensity of staining ($p = 0.98$) with severity of the dysplasia in the biopsy (Figure 1c). The PR LI was 0 in three biopsies with normal epithelium, two with MiD/MoD, four with SD, one with CIS, two with MIC, and three areas of normal epithelium adjacent to the dysplasia (Table 1). There was no significant relation of the PR LI or intensity of staining in normal epithelium with the severity of the adjacent dysplasia, the presence of HPV 16, other hr-HPV genotypes, or multiple hr-HPV genotypes in the biopsy (Table 1, Figure 2c).

ER/MIB-1 and PR/MIB-1 ratios

Figure 3 shows a highly significant decrease ($p < 0.001$) of the ER LI/MIB-1 LI ratio (Figure 3a) and the ER intensity of staining/MIB-1 LI ratio (Figure 3b) with increasing severity of the dysplasia. The PR intensity of staining/MIB-1 LI ratio (Figure 3b) also shows a significant decrease ($p = 0.019$) with increasing severity of the dysplasia, but this was not observed for the PR LI/MIB-1 LI ratio (Figure 3a). In normal epithelium directly adjacent to the dysplasia no alterations in the ratios with increasing severity of the adjacent dysplasia were observed (Figures 3c and 3d).

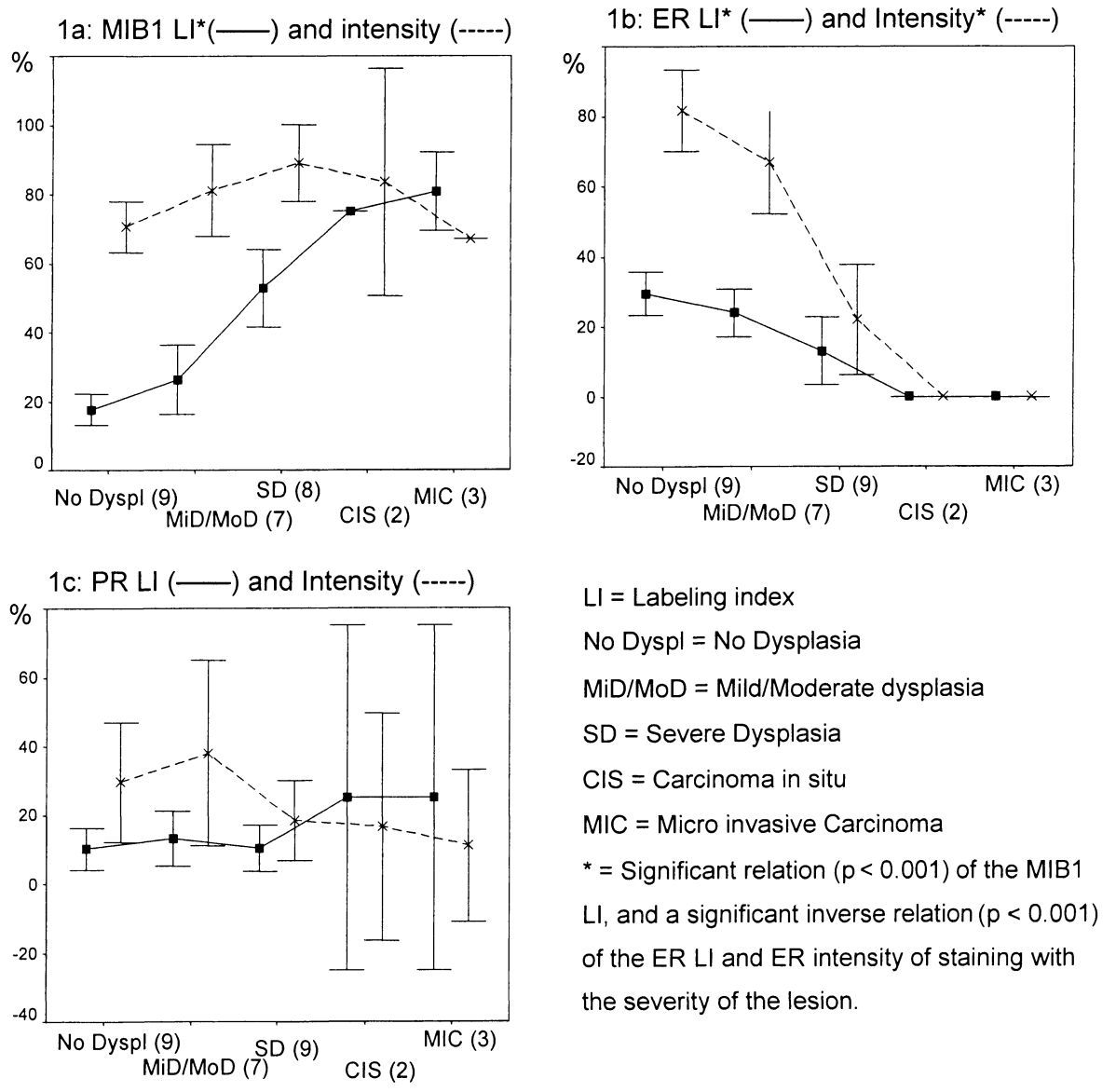


Figure 1. — MIB-1-, ER-, and PR-LI (■) and intensity of staining (x) in relation to the severity of the dysplasia in the biopsy (Vertical bars represent 2 standard errors of the mean).

Discussion

This study confirms a significant increase in MIB-1 LI with increasing severity of cervical dysplasia, as described previously [9-11], while no relation between the MIB-1 intensity of staining and severity of the dysplasia was observed.

The ER LI and intensity of staining showed a significant inverse relation with the severity of the dysplasia in this study. In the literature, some studies showed a down regulation of ER expression in dysplasia in relation to HPV 16 or 18 infections and less with HPV 31, 33, or 35 [8, 16, 17], while others did not find a relation between hr-HPV infections and ER expression at all [14, 18, 20].

Normally, a variation in ER (and PR) expression in relation to hormonal fluctuations can be observed in normal cervical epithelium. ER is expressed in the basal cells of the epithelium throughout the menstrual cycle, while the ER expression in parabasal cells is higher in the follicular phase and less in the luteal phase or during pregnancy [8]. Indeed, high levels of progesterone (pregnancy, luteal phase) have been shown to decrease ER expression in the endometrium and myometrium, and inhibit the proliferative effect of estrogens on the epithelium [26, 27]. Furthermore, estrogens that bind the ER inhibit its further expression, while simultaneously the synthesis of the ER and PR is stimulated [26, 27]. The observed association between a higher proliferative activity with a decreased ER expression in cervical epithelium during the luteal phase of the menstrual cycle suggests a regulation of the proliferation of normal cervical epithe-

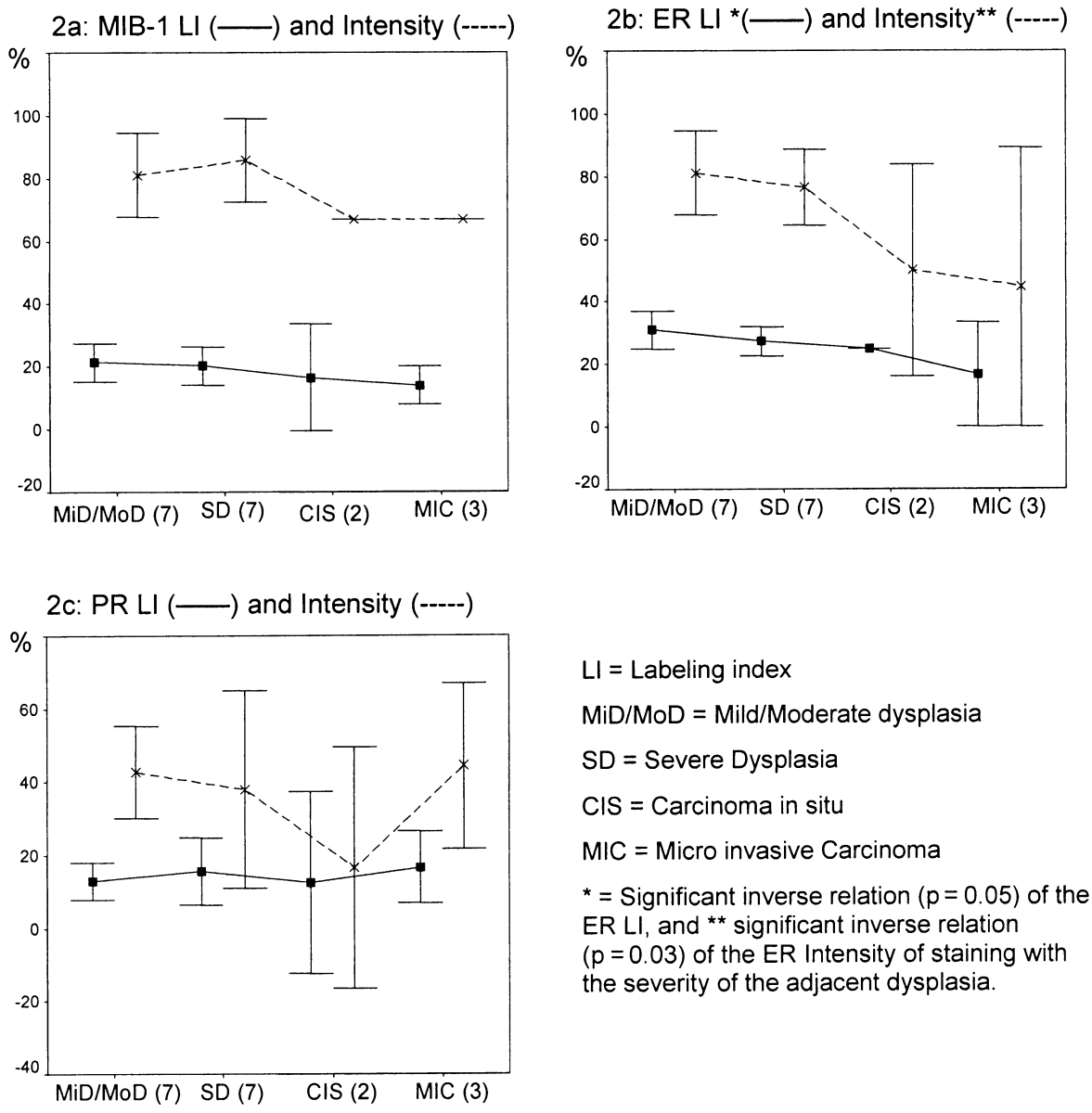
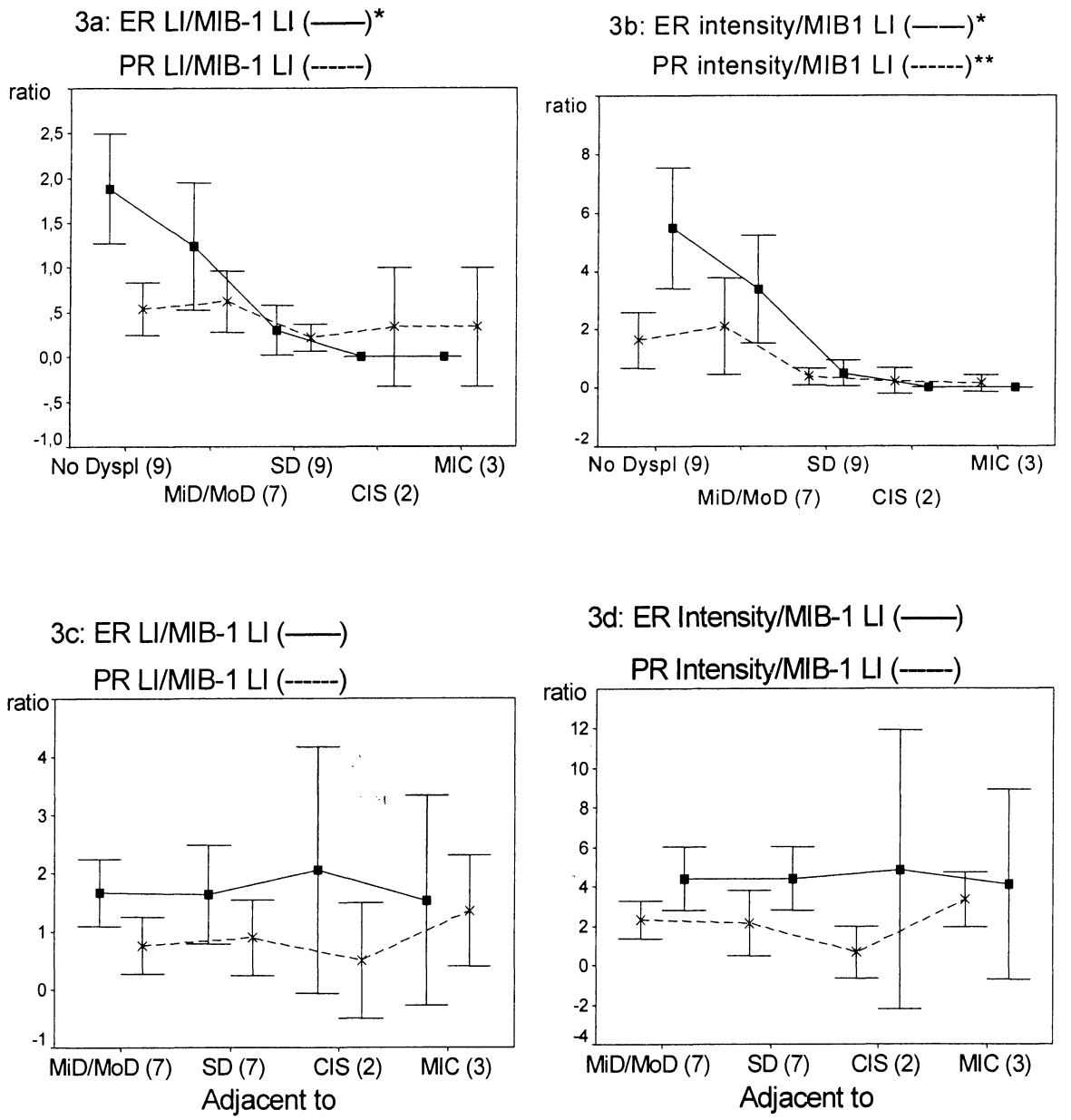


Figure 2. — MIB-1-, ER-, and PR-LI (■) and intensity of staining (x) in normal epithelium adjacent to the dysplasia in relation to the severity of that dysplasia. (Vertical bars represent 2 standard errors of the mean).

lium by sex steroid hormones [8, 17]. Down regulation of ER expression in dysplasia, as observed in this study, indicates that malignant transformation of cervical epithelium is associated with loss of normal growth control by sex steroid hormones [8, 17]. Indeed, Bulten *et al.* found in their study of atypical atrophic postmenopausal cervical scrapes that estrogen therapy did not alter the proliferative activity in patients with high-grade CIN, while there was a significant change in proliferative activity in patients without CIN [28].

The PR LI and intensity of staining did not show any relation with the severity of the dysplasia in this study. One study described a significant relationship between the degree of dysplasia and PR expression especially in HPV 16/18 infected lesions, suggesting that progesterone is a co-factor in HPV-mediated cervical neoplasia [15]. Others described a large variation in PR expression in dysplastic lesions as well as in invasive cervical cancer, and like this study, did not confirm this relation [8, 14, 17]. Normally, PR expression is induced by estrogens and decreased by progestatives [26]. The fact that the majority of the patients in this study underwent colposcopy with biopsies during the luteal phase, or under the influence of oral progestatives, may have caused the low expression of the PR, but does not in our opinion explain the lack of any relation between the PR and dysplasia. Recently, two iso-forms of the PR have been found, which are each associated with different proteins that are impor-



* $p < 0.001$; ** $p = 0.019$

Figure 3. — ER LI/MIB-1 LI-, PR LI/MIB-1 LI-, ER intensity/MIB-1 LI-, and PR intensity/MIB-1 LI-ratio in relation with the severity of the dysplasia (Figures 3a and 3b), and in normal epithelium in relation to the adjacent dysplasia (Figures 3c and 3d). Vertical bars represent 2 standard errors of the mean.

tant in its response to sex steroid hormones [26]. Whether these iso-forms are responsible for the large variation in the detection of PR expression in normal and dysplastic cervical lesions needs further study.

The lack of any relation of MIB-1, ER-, and PR-expression in dysplastic lesions with the presence of other more or less severe dysplastic lesions within the same cervix may indicate that different dysplastic lesions develop relatively independently from each other under the influence of the same or different hr-HPV genotypes [25, 29, 30]. The relation between the detection of single and/or multiple hr-HPV genotypes within a single cervix or within a single lesion of these patients has been described and discussed in detail previously [25].

The ER LI and ER intensity of staining in normal epithelium directly adjacent to the dysplasia showed a significant inverse relation with the severity of that dysplasia, while this was not observed for the MIB-1 LI, PR

LI, or PR intensity of staining. Whether the adjacent dysplastic lesion (hyperproliferation) is the cause of the lower ER expression in adjacent normal epithelium, or whether this is caused by other factors, like for instance infection with hr-HPV, needs further study.

The significant inverse relation of the ER/MIB-1 and PR/MIB-1 ratios with increasing severity of the dysplasia indicates that there is a progressive loss of control of the proliferative activity by sex steroid hormones. In normal epithelium adjacent to the dysplasia, no such relation was observed, indicating that hormonal sensitivity is still present, despite the lower ER expression in these areas. Down regulation of ER expression in normal epithelium adjacent to dysplasia may in this respect be one of the first alterations that take place in the development of cervical dysplasia in women infected with hr-HPV. Due to the small number of biopsies and patients in this study, larger studies are needed to confirm these data and to investigate the clinical significance of these findings.

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