CHAPTER: 6

IMMUNOHISTOCHEMISTRY
CHAPTER 6: IMMUNO-HISTOCHEMISTRY

6.1: INTRODUCTION

The most commonly used stain in histopathology is a combination of hematoxylin and eosin (often abbreviated H&E). Hematoxylin is used to stain nuclei blue, while eosin stains cytoplasm and the extracellular connective tissue matrix pink. Other compounds used to colour tissue sections include safranin, oil red O, congo red, silver salts and artificial dyes.

Histochemistry refers to the science of using chemical reactions between laboratory chemicals and components within tissue. Recently, antibodies have been used to stain particular proteins, lipids and carbohydrates and which is known as immunohistochemistry. This technique has greatly increased the ability to specially identify categories of cells under a microscope. Other advanced techniques include in situ hybridization to identify specific DNA or RNA molecules. These antibody staining methods often require the use of frozen section histology (Michael et al. 2005).
6.2: REVIEW OF LITERATURE

A Brazilian study:

A total of 24 normal tissues, 17 cases of cervical intraepithelial neoplasia and 7 of invasive carcinomas were studied. The ER/PR ratio was determined by immunohistochemistry using monoclonal antibodies specific for each receptor. Adjacent tissue slides were submitted to generic PCR for human papillomavirus (HPV) DNA detection followed by typing by dot blot hybridization. About half (45.8%) of the tumors were HPV DNA positive while 29.1% of the patients were also HPV positive in their respective normal tissue.

ER was negative in the tumoral epithelium of 11 HPV-positive patients. There was a trend in the ER distribution in normal tissue that was opposite to that from lesions, but it was not statistically significant. No difference in ER distribution in stromal tissues was observed between HPV-positive and HPV-negative tissues. PR staining was negative in the epithelium of all cases studied. The results obtained from this small number of cases cannot be considered to be conclusive but do suggest that factors related to viral infection affect the expression of these ER/PR cervix receptors.

Human papillomaviruses (HPVs) are commonly found in the genital tract of sexually active women and studies have confirmed that persistent infection with high-risk types of HPV in the cervix precedes cervical cancer (Shiffman et. al, 1993; Villa, 1997).

However, although HPV is a necessary cause it cannot be considered sufficient to trigger all the changes required to lead to this neoplasia (Franco et al., 1999). Mutagenic and immunosuppressive agents
can cooperate with HPV through different mechanisms Villa, et al., 1997; Coelho et al., 2004. Interaction is known to occur between HPV and some types of hormones, particularly estrogens, and their antagonists, resulting in the alteration of viral gene expression (Chen et al., 1996). It is known that the upstream regulatory region of several HPVs contains glucocorticoidresponsive elements (Chan et al., 1989).

These hormones have a receptor similar to that of the estrogen receptor (ER) hormone Mader et al., 1989; Picard, 1990). A series of reports have shown that mean ER positivity in the cervical mucosa can vary from 13 to 100%. For progesterone receptors (PR) the variation is even wider, i.e., 0 to 100%. Tissues from the uterine cervix have been reported to be PR positive (Monsonego, 1991; Masood, et al., 1993; Kim et al., 1992; Robertson et al., 1993; Keng et al., 1994). No statistically significant correlations have been found for the ER/PR ratio in the cervix when related to menstrual cycle, menopause, histological type or ploidy analysis (Monsonego, 1991; Masood et al., 1993; Kim et al., 1992; Robertson et al., 1993). Mosny, et al., (1989) demonstrated a gradient of ER detection in the uterine cervix: while a low expression of ER was observed in moderate dysplasia, cases of severe dysplasia and cervical cancer were negative for the receptor.

One major explanation for these discrepancies could be related to the location of the receptors, which can be found either in the epithelium or in the stroma, to the size of the tissue analyzed, and to the method for receptor detection (Monsonego, 1991; Masood, et al., 1993; Mosny et al., 1989; Taylor, 1996).

In an attempt to clarify some of these issues it was sought to evaluate the concentration of ER and PR in samples from the normal
uterine cervix, from cervical intraepithelial neoplasia (CIN 1, 2 and 3) and from invasive carcinoma, associated or not with the presence of HPV DNA. Consecutive cases of CIN and invasive carcinomas of the uterine cervix were obtained at the Gynecology Department of the Hospital do Câncer, Fundação Antônio Prudente, São Paulo, Brazil, between May 1996 and March 1997.

The following samples were obtained: 5 CIN 1, 3 CIN 2, 9 CIN 3, and 7 cervical invasive carcinomas (4 squamous cell carcinomas and 3 adenocarcinomas). Patient age ranged from 23 to 50 years (mean: 39 years).

The following eligibility criteria were established for patient inclusion in the study: premenopausal women who had not used oral contraceptives for the last 2 years and a histologically confirmed diagnosis of CIN and invasive carcinoma, not previously treated. A fragment of tumoral tissue was removed with a 5-mm diameter disposable biopsy punch (Stiefel Company, Coral Gables, FL, USA). This collection device was chosen to reduce the collection of adjacent stromal tissue.

Stiefel Company, Coral Gables, FL, USA searched for macroscopically normal tissue close to the tumoral area and obtained a second tissue fragment with another biopsy punch to avoid any possible tissue cross-contamination. Each of the tissues was divided into three equal parts, resulting in six fragments. Two of the fragments corresponding to either normal or abnormal tissues were immediately placed in liquid nitrogen, followed by storage at –70°C for variable periods of time before being tested for HPV DNA.
The third fragment was immersed in buffered formalin for routine histopathological procedures and immunohistochemical determination of ER and PR. DNA was extracted from the tissue by a routine procedure (Rapid Prep. Genomic Isolation, Pharmacia Biotech, Uppsala, Sweden). DNA amplification was performed using the polymerase chain reaction (PCR) according to previously described protocols employing generic primers MY09/MY11 capable of amplifying a fragment of the L1 gene of all known genital HPVs (Bauer et al., 1992).

Reactions were processed in a thermal cycler (Perkin-Elmer Cetus, Foster City, CA, USA). HPV typing was performed by dot blot hybridization as previously described. This procedure allows the identification of more than 30 HPV types. In 4 cases, the PCR products were further typed by restriction fragment length polymorphism (Bernard, 1994). Positive and negative reaction controls were always used in the PCR assays. As an internal control for DNA quality the amplified fragment of the β-globin gene and the reaction was positive for 100% of the specimens. The tissues were submitted to standard immuno 85 Braz J Med Biol Res 37(1) 2004 Estrogen and progesterone receptors in cervical neoplasia histochemical evaluation. The monoclonal antibody used for ER was the 6F11 clone (NCL-ER - 6F11, Novocastra, UK) diluted 1/20. The monoclonal antibody, clone 1A6 (NCL-PGR, Novocastra, UK), also diluted 1/20, was used for PR determination. Positive and negative controls were used in all assays. The percentage of positive cells (nuclear positivity only) was determined in the stroma, as well as in the epithelium, normal mucosa, atypical epithelium (CIN 1, CIN 2 and CIN 3), and invasive carcinoma. A differential cell counter (Diffcount, model MD-8, Modulus Data System, Santa Clara, CA, USA)
was used. In all cases, a minimum of 100 and a maximum of 200 cells were counted, and the results are reported as frequency of positive cells.

Since normal tissue, considered as a control, was removed from the same patient, they also used the McNemar test (Thompson EA, 1990). Among 24 patients, HPV DNA was detected in 11 (45.8%) cases with cervical lesions (CIN and cancer), while 7 (29%) samples were positive in the control tissues. HPV16 was the most frequent type, being present in 12 tissue samples (8 CIN and cancer and 4 control tissues), followed by 4 type 58 positive samples (2 CIN and their controls), and finally by type 33, present in one invasive carcinoma and in its respective control. As expected it was observed a gradient of HPV positivity ranging from 29.2% for normal tissue to 35.3% for CIN 1, 2, and 3 together and to 71.4% for invasive carcinomas. These frequencies are somewhat lower than the previous reports in which almost 100% of the invasive cervical cancers bear HPV-DNA sequences (Walboomers, 1999). The low HPV positivity observed could be due to the limited amount of tissue available, although the globin control of all specimens was positive. One should also consider that low-grade CINs tend to be heterogeneous and it is possible that the fragment submitted to PCR may not contain HPV-positive cells. Nevertheless, in the present study there was no case in which the tumoral tissue was HPV DNA negative and the adjacent normal tissue was HPV positive; moreover, the same HPV types were found in both tumor and control tissues. This could be explained by the physical proximity of the two biopsies, although extreme care was taken to avoid cross-contamination at the time of sample collection and processing.
The expression of ER and PR was analyzed both in the epithelium and in the stroma of normal and tumoral tissues. They observed that 20.8% of the tumors (5 of 24 cases of CIN and invasive cancer) were positive for ER expression in the epithelium. On the other hand, half of the adjacent normal tissue samples (12 of 24) were positive for ER.

Furthermore, the distribution of ER in the stroma was identical for normal and tumoral tissues with positivity ranging from 20.8 to 25% in the tissues studied (P=1.000). Similar results were obtained for PR present in the stroma of both normal and tumoral tissues. Interestingly, PR expression was not observed in the epithelium of any case studied and all 3 adenocarcinomas studied were both ER and PR negative.

Stratification on the basis of the HPV result revealed that all HPV-positive CIN and invasive cancers were negative for ER expression in the epithelium. Conversely, HPV-negative tumours expressed ER in 38.4% of cases (5 of 13; A similar inverse distribution of epithelial ER was observed in normal tissues. Expression of ER in stromal cells was similar for HPV-positive and -negative tumors. On the other hand, an increased number of ER was observed in the stroma of normal tissue, i.e., 5 ER-positive cases of 7 HPV-positive cases (71.4%). Once more, in HPV-negative normal tissues ER positivity was 41.1% Coelho et al.,(2004).

There was no statistically significant difference in the frequency of detection of HPV DNA between tumoral and normal tissues.

Studies carried out to assess the presence of ER and PR in the uterine cervix have reported different results. These variations may have resulted from differences between the patients and/or the methodology used for the evaluation of the receptors. They chose an
assay based on immunocytochemistry because of its easy application to small tissue fragments, consequently avoiding problems related to the possibility of mixing epithelial and stromal cells. Cellular heterogeneity, not taken into consideration in most studies, may account for the variation in ER/PR concentration in the tumor or normal epithelium or even in the subjacent stroma (Mosny et al., 1989).

It was sought to control for this variation by sampling both tumoral and normal tissues from the same patient at the same time, therefore achieving a very good homogeneity of the samples submitted to comparison.

However, one may argue that due to the same embryologic origin and proximity of the tumor, the normal counterpart considered here as control was probably exposed to the same factors that may have caused the lesion.

Nevertheless, the differences observed in the expression of these steroid receptors are reassuring. ER expression in the epithelium was observed in only 20% of HPV-positive tumors, as compared to 50% of the normal tissues. This result is similar to that obtained in previous studies in which ER expression was found to be inversely associated with disease status (Monsonego, et al., 1991; Masood et al., 1993; Kim et al., 1992; Robertson et al., 1993; Keng et al., 1994). On the other hand, a similar ER distribution was observed in the stroma of both tumors and normal tissues. This trend, although not statistically significant, may be related to the fact that HPV replicates exclusively in the epithelial layer. In fact, the distribution of epithelial ER in the study presented greater negativity in the lesions restricted to the epithelial layer (CIN 1, CIN 2, and CIN 3).
The 100% ER negativity in the epithelium of invasive carcinomas detected in the sample leads us to speculate that the invasive lesions "contaminate" the subjacent stromal tissue, which then will primarily consist of tumoral cells. When the tissue fragment to be analyzed is small, as in the study, this aspect of predominance of tumoral cells in stromal tissue may contribute to ER negativity.

Negative results concerning PR expression in the epithelium of both tumoral and normal tissues contrast with previous reports (Kim, et al., 1992; Robertson et al., 1993; Keng et al., 1994). It was excluded a technical problem, since the stroma of the same cases reacted positively. Recent studies using an enzyme-linked immunoassay have shown a 74 to 100% variation in PR positivity in the normal uterine cervix (Kim et al., 1992; Robertson et al., 1993; Keng et al., 1994).

Moreover, PR is expressed at high levels in low and high grade CIN lesions, whereas:

Association between human papillomavirus (HPV) and estrogen receptor in the epithelium of cervical intraepithelial neoplasias and invasive cervical cancers.

**Estrogen negative positive total receptor:**

<table>
<thead>
<tr>
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<th>Negative</th>
<th>Positive</th>
<th>Total</th>
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<tbody>
<tr>
<td></td>
<td>8 (61.5%)</td>
<td>5 (38.4%)</td>
<td>13 (100%)</td>
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<tr>
<td></td>
<td>11 (100%)</td>
<td>0 (0%)</td>
<td>11 (100%)</td>
</tr>
<tr>
<td></td>
<td>19 (79.1%)</td>
<td>5 (20.8%)</td>
<td>24 (100%)</td>
</tr>
</tbody>
</table>

A statistically significant association was detected.

Association between human papillomavirus (HPV) and estrogen receptors in the normal epithelium of the uterine cervix.
Estrogen negative positive total receptor:
Negative 10 (58.8%) 2 (28.5%) 12 (50%)
Positive 7 (41.1%) 5 (71.4%) 12 (50%)
Total 17 (100%) 7 (100%) 24 (100%)

There was no statistically significant association

Braz J Med Biol Res 37(1) 2004 Estrogen and progesterone receptors in cervical neoplasia very low levels of this receptor are observed in invasive cervical carcinomas (Monsonego et al., 1991). Since PR assembly is an estrogen-dependent process, the presence of functional ER can be considered to be necessary for the formation of PR (Masood et al., 1993).

In their study, all HPV-positive tumors were negative for epithelial expression of ER, while about 38% of the HPV negative cases were ER positive. The transcriptional activation of the human steroid hormone receptors triggers a series of molecular events that ultimately affect the estrogen - or progesterone - responsive elements present in different target genes. The transcription of HPV genes, some of which code for products with transforming activity, is dependent on the regulatory elements present in the upstream regulatory region (Chan et al., 1989). One of this transcription factor binding sites is a glucocorticoid-responsive element found in genital HPVs, but not in cutaneous HPV types (Chan et al, 1989). This element responds to both progesterones and glucocorticoids by increasing the transcription of the E6 and E7 genes of HPV 16 and 8, two of the more prevalent high-risk HPV types (Chan et al., 1989).

These data suggest an interaction between HPV and steroid hormones to promote cell proliferation, eventually leading to cell transformation.
The transformation zone of the uterine cervix where more than 90% of HPV-induced lesions and cervical cancers occur is also the genital site most sensitive to estrogens (Elson et al., 2000). Epidemiological data suggest that the use of oral contraceptives may increase the risk of cervical neoplasia (Moreno et al., 2002). Taken together with the biological evidence, these data indicate that young women taking oral contraceptives are exposed to a higher risk of developing cervical neoplasias, especially if they were also exposed to high-risk HPVs.

What is not clear, however, is how the regulation of the expression of steroid receptors may interfere with this process. Additional studies with a larger number of patients will be necessary to establish the relevance of the association between hormone receptors and HPV-induced lesions in the uterine cervix.
6.3: MATERIALS AND METHODS

Estrogen (ER) and progesterone (PR) receptors in the normal uterine cervix, cervical intraepithelial neoplasia and invasive carcinoma is studied in 30 cases of carcinoma cervix and few cases in cervical dysplasia cases in Silchar Medical College & Hospital, Silchar and Lalpath Laboratories, New Delhi. Tissue was collected by removing a fragment of the tumoral area using a 5-mm diameter biopsy punch, followed by removal of a macroscopically normal area as close as possible from the tumour. Histopathological confirmation was obtained for all specimens and immunohistochemistry was done using specific monoclonal antibodies specific for estrogen and progesterone receptors.
6.4: RESULTS AND DISCUSSION

Table 6.1: Study of ER PR in cervical carcinoma in 30 cases (30-50 years of age)

<table>
<thead>
<tr>
<th>Normal cervix</th>
<th>Squamous cell carcinoma</th>
<th>Adeno-carcinoma</th>
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<tbody>
<tr>
<td></td>
<td>Well differentiated</td>
<td></td>
</tr>
<tr>
<td>05</td>
<td>05</td>
<td>05</td>
</tr>
<tr>
<td></td>
<td>Moderately Differentiated</td>
<td></td>
</tr>
<tr>
<td></td>
<td>03</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Poorly differentiated</td>
<td></td>
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<tr>
<td></td>
<td>03</td>
<td>04</td>
</tr>
</tbody>
</table>

ER + 3 cases | ER = 02 | ER + 02 | ER(-Ve) | ER + 3 | ER (-Ve) |
PR + 1 case  | PR = 02 | PR (- Ve) | PR-Ve | Pr (-Ve) |
ER+PR(-Ve) 1 case | ER+PR=- 01 | ER+PR=- 01 | ER&PR (-Ve) | ER & PR (-Ve) |

6.4: DISCUSSION

Mosny, et al., (1989) also demonstrated a low expression of ER and PR in moderate dysplasia, in severe dysplasia. And in advance cervical cancer with poor differentiation both ER PR is down regulated.
6.5: CONCLUSION

In ER & PR study there is no definite relation of ER & PR in normal cervical tissue and carcinoma growth. But there is a definite correlation that both ER & PR is negative in poorly differentiated squamous cell carcinoma and adenocarcinoma. So it can be used as a prognostic and outcome value in cervical carcinoma.
Fig. 6 (a) : Immunohistochemistry picture showing a poorly differentiated squamous cell carcinoma with down regulation of estrogen receptors (ER)

Fig. 6 (b) Immunohistochemistry picture showing a poorly differentiated squamous cell carcinoma with down regulation of progesterone receptors (PR)
Fig. 6 (c): Immuno histochemistry picture of LSIL with down regulation of PR (Negative) in both the picture.
6.6: BIBLIOGRAPHY


