

IL10 PRODUCTION IN CULTURES OF LYMPHOCYTES DERIVED FROM BIOPSIES OF NORMAL EXOCERVIX, TRANSFORMATION ZONE AND SQUAMOUS INTRAEPITHELIAL LESION.

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SUMMARY

The immune system appears to play a role in the development of squamous intraepithelial lesions (SIL) and their progression to cancer. We have developed a technique to isolate and cultivate lymphocytes either from the epithelium or from the underlying stroma of small biopsies from the normal exocervix, the transformation zone (TZ) and SIL. The majority of cells derived from the epithelium of all biopsies were CD8⁺ T cells. No major difference was observed for the lymphocyte phenotype. Among all cytokines tested by ELISA (IL10, IL4 and IFN γ), only IL10 was significantly higher in the TZ in comparison with the exocervix. In cultures derived from the stroma, a decreased percentage in T cells was observed in the TZ and SIL in comparison with the exocervix. This decrease in T cells concerned CD8⁺ (SIL) and TCR $\gamma\delta$ ⁺ T cells (TZ and SIL). We did not observe any difference in IL10 or IL4 production, although patients with SIL produced more IFN γ . The higher levels of IL10 production by lymphocytes derived from the epithelium of the TZ might contribute to the predisposition of this region to cervical carcinogenesis.

INTRODUCTION

Uterine cervix cancer represents one of the best examples of human malignant neoplasm preceded by well characterized preneoplastic stages which are named as squamous intraepithelial lesions (SIL). Moreover, the most important etiological agent of this cancer, the human papillomavirus (HPV), has been well characterized. However, HPV infection is not sufficient for cancer development. Considering the small number of infected individuals who eventually develop cervical cancer and the long latency period between primary infections and the onset of cancer, it appears that other promoting factors are involved in malignant progression. In this context, the local immune status within the transformation zone (TZ) of the cervix, an area of metaplastic squamous epithelium, where the majority of intraepithelial and invasive neoplasms develop, might play a role in the outcome of HPV infection and associated-(pre)cancerous lesions, as suggested by the observation that these cancers are more frequent in immunodeficient women (Benton et al., 1992; Petry et al., 1994; Calore et al., 1998). Type II (IL4/IL6) or immunosuppressive (IL10) cytokines are preferentially found in the cervix (Al-Saleh et al. 1998; Giannini et al. 1998) and in the peripheral blood (Jacobs et al. 1998). Our previous immunohistochemical studies have suggested that CD4⁺ lymphocytes are responsible for the type II cytokine production in SIL (Al-Saleh et al, 1998). Other studies focusing on the status of the local immune response, have shown that the clearance of cervical HPV infection, either spontaneously or after treatment with IFN γ and IFN α , is associated with a type I pattern of local cytokine production (Arany and Tying 1996; Scott et al. 1999).

In order to characterize the SIL infiltrating lymphocytes and to determine their role in the local immune response, we have developed protocols to isolate lymphocytes from the epithelium and stroma of SIL and normal exocervix biopsies and to cultivate them *in vitro*. In addition, we have also analyzed the lymphocytes from the TZ and exocervix biopsies of hysterectomized patients without cervical lesion. Due

to the small size of the biopsies (2-4 mm), long-term cultures were necessary to obtain sufficient lymphocyte numbers. The originality of our culture method is that the lymphocytes from the epithelium and the stroma were separately isolated and cultivated (Jacobs et al, 1999).

MATERIALS AND METHODS

Patients. Patients with a cervical SIL diagnosed by cytology and a colposcopically-directed biopsy were recruited for this study. For the *in vitro* culture experiments fresh biopsies (2-4 mm³) were obtained before any surgical procedures. These biopsies were carried in keratinocyte culture medium (DMEM/HAMs) (Rheinwald and Green, 1975) containing 100 U/ml gentamycin (GIBCO, Belgium) and 1.5 µg/ml fungizone (GIBCO) and processed within a few hours. This study protocol was approved by the Ethics Committee of the Faculty of Medicine at the University of Liège.

Cell Cultures. The method for cell isolation and culture was previously described (Jacobs et al., 1999). For each patient, lymphocytes derived from a normal exocervix and from TZ or from SIL biopsies were analyzed

PCR. HPV DNA was detected in biopsy specimens by PCR with degenerated oligonucleotides hybridizing in L1 open reading frame (Jacobs et al., 1995)

Phenotypic analysis. Double- and triple-staining were performed with fluorescent conjugated antibodies. The following monoclonal antibodies were used: anti-CD3 (PerCP), CD4 (FITC), CD8 (PerCP), CD19 (PerCP), CD56 (PE) and TCRγδ (FITC) (Becton Dickinson, Belgium) and anti-CD16 (FITC) (Ortho, R, NJ). The phenotype was performed on 1x10⁵-5x10⁵ cells following standard protocols. The cells were analysed for fluorescence intensity with a FACScan (Becton Dickinson).

ELISA assays. The cytokines IL-4, and IL-10, were measured by using specific immunoassays from Pharmingen (Belgium). For IFNγ ELISA assay, antibodies from Serpine (Belgium) were used. Recombinant human IL-4, IL-10 and IFNγ were used as reference standards.

RESULTS

Phenotype and cytokine production of lymphocytes derived from the epithelium.

HPV detection was performed by PCR in all the biopsies. HPV⁻ biopsies of patients with normal cervix and HPV⁺ SIL biopsies of patients with SIL were analyzed in this work. For patients with SIL some exocervix biopsies were HPV⁺. Since lymphocytes are rare in the epithelium, irradiated autologous peripheral blood mononuclear cells (PBMC) and phytohematagglutinin (PHA) were necessary for lymphocyte expansion in the presence of IL2. After 20-30 days of culture, 2X10⁶ to 100X10⁶ cells were obtained per biopsy. These numbers were similar for all types of biopsies (normal exocervix, normal TZ or SIL biopsies) (data not shown).

In all cultures (exocervix, TZ and SIL), most of cells were CD8⁺ T cells (Figure 1). Few NK cells were detected and no B cells (CD19⁺) were observed (data not shown). A smaller percentage of CD8⁺ T cells and a higher percentage of TCRγδ⁺ cells were found in cultures derived from the epithelium of patients with SIL (both in the normal and the pathological part of the cervix) in comparison with normal women (Figure 1). The presence of HPV in some cultures of exocervix from patients with SIL could explain the difference observed between both exocervical cultures. However a larger number of samples is necessary to establish a correlation between the presence of HPV and the phenotype of lymphocytes.

IL10 (immunosuppressive cytokine), IL4 (type II cytokine) and IFNγ (type I cytokine) were analyzed in the culture supernatants by ELISA (Figure 2). No major differences were observed between exocervix, TZ and SIL, except for a significantly higher level of IL10 in cultures of lymphocytes derived from TZ in comparison with the exocervix. This result suggests that lymphocytes could be responsible for the increase in IL10 mRNA levels detected in the TZ by rt-PCR (Giannini et al., 1998). IFNγ production in cultures from patients with SIL seemed to be higher than in cultures of patients without SIL. As IFNγ

production is induced in response to viral infection, the presence of HPV could explain the higher production of IFN γ .

Phenotype and cytokine production of lymphocytes derived from the stroma.

In order to develop a model reminiscent of the *in vivo* situation, lymphocytes were obtained in the presence of IL2 alone, thus without addition of PHA and autologous PBMC. Stroma tissue fragments were put in medium containing IL2 (50 U/ml) and the lymphocytes migrating out of the tissue were collected. An average of 0.8×10^6 lymphocytes and 1.6×10^6 lymphocytes were obtained after 20 and 30 days of culture respectively. The numbers of lymphocytes generated in culture were similar in all types of biopsies (normal exocervix, normal TZ or SIL biopsies) (data not shown).

After 20-25 days of culture, a lower percentage of T cells was obtained in stromal cultures from TZ as compared to normal exocervix (Figure 3). This decrease was more pronounced and statistically significant when we compared cultures derived from SIL with exocervical cultures. CD3⁺ cells were CD56⁺ NK cells, some of these cells expressed CD8 (Figure 3). This increased proportion of NK cells was also observed in cultures derived from the TZ (data not shown). Very few CD16⁺ NK cells were detected in the cultures (data not shown). The populations of T cells with a cytotoxic phenotype (CD3⁺CD8⁺) and TCR $\gamma\delta$ ⁺ T lymphocytes, which can be considered as a first line of defense (Boismenu and Havran, 1997), were found to be under-represented in SIL, as compared with normal exocervix (Figure 3). The lower proportion of CD8⁺ T cells and TCR $\gamma\delta$ ⁺ cells in cultures of SIL underlying stroma could be due to the smaller number of these cells *in situ* or to a proliferative defect in response to IL2. However, immunohistochemistry data have shown previously a decrease in the CD8⁺ T cell numbers in the stroma adjacent to the SIL (Al-Saleh, unpublished data). We also observed a decreased percentage of TCR $\gamma\delta$ ⁺ lymphocytes in the TZ in comparison with the exocervix in 6/7 cultures (data not shown). These data suggest that the modifications of lymphocyte populations observed in SIL cultures could be related in part to the particular phenotype of lymphocytes infiltrating the TZ, in addition to alterations caused by HPV infections and associated lesions.

In order to directly compare the phenotype of stroma-derived with the epithelium-derived lymphocytes, we generated lymphocyte cultures from the stroma using the same culture conditions as for the epithelium (IL2+ PHA + autologous irradiated PBMC). With these culture conditions, we observed a decreased proportion of NK cells in the cultures with autologous PBMC (data not shown). We have not yet evaluated enough samples to determine if the differences in the percentage of CD8⁺ and TCR $\gamma\delta$ ⁺ cells are maintained under these conditions (data not shown).

The cytokines studied in cultures derived from the epithelium were also analyzed in cultures derived from the stroma in the presence of IL2 alone (Figure 4). No significant difference was detected between the exocervix versus TZ cultures and between the exocervix versus SIL. However, an increased production of cytokines, especially of IFN γ , were observed in patients with SIL in comparison with patients with normal cervix (Figure 4). As IFN γ production is induced in response to virus infection, the higher production of IFN γ , in some patients, could be a reflection of an immune response against HPV.

CONCLUSIONS

We found that effector cells, such as CD8⁺ T cells and TCR $\gamma\delta$ ⁺ T cells, are underrepresented in cultures derived for the SIL underlying stroma. We also observed a decreased proportion of TCR $\gamma\delta$ ⁺ T lymphocytes in TZ underlying stroma as compared with exocervical cultures. These results suggest that cellular immune alterations might be involved in the development and progression of SIL. Moreover, the immunosuppressive cytokine IL10 has been shown to be highly produced in lymphocyte cultures derived from TZ epithelium suggesting that lymphocytes are probably the main source of the previously reported increased expression of this cytokine in the TZ *in vivo* (Giannini et al., 1998).

The high production of IL10 associated with the smaller percentage of TCR $\gamma\delta^+$ T cells in TZ cultures may participate to the persistence of HPV infection and to the predisposition of this region to cervical carcinogenesis.

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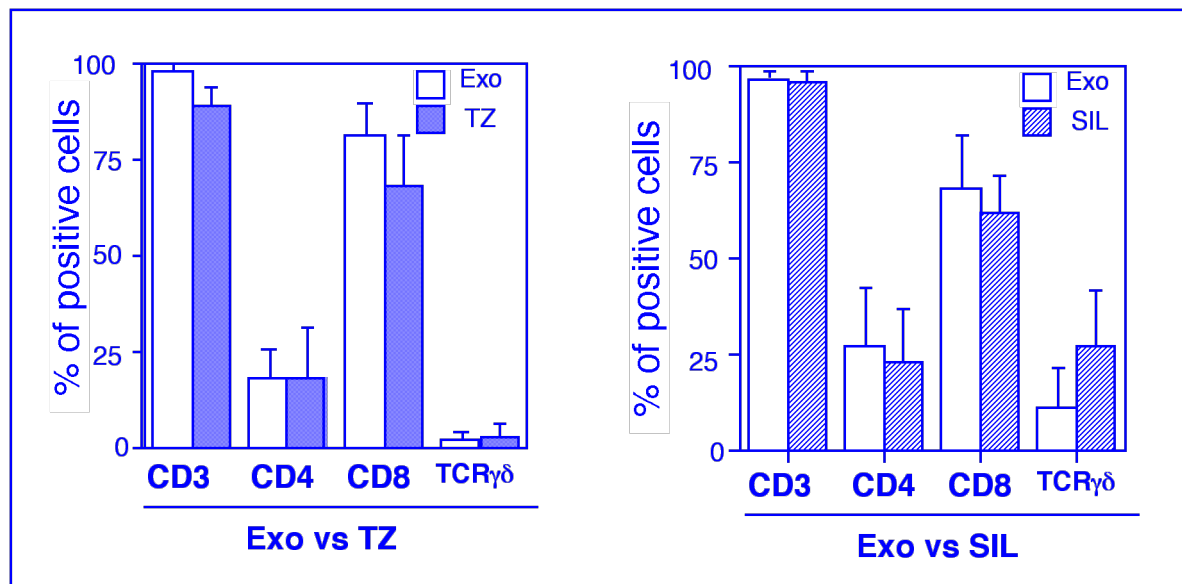


Figure 1: Phenotype of lymphocytes derived from the epithelium. Exo and TZ = normal exocervix and transformation zone biopsies of patients with normal cervix; Exo and SIL = normal exocervix and SIL biopsies of patients with SIL. Means \pm SE after 20-25 days of culture ($n \geq 8$) are represented.

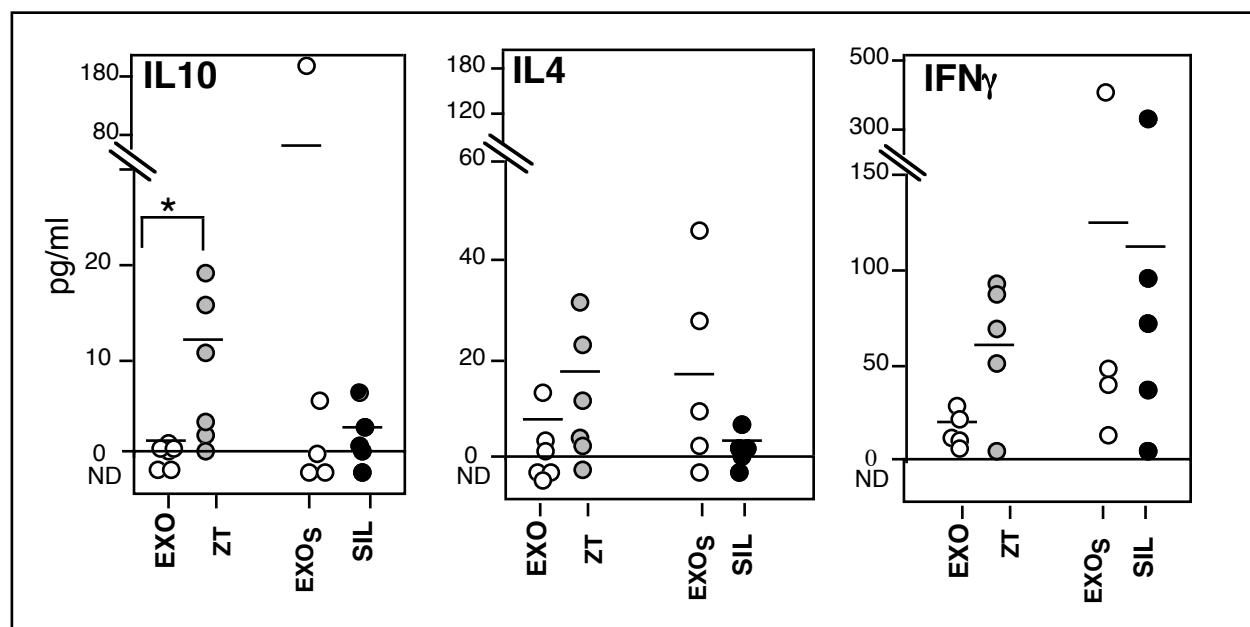


Figure 2 Cytokine production in cultures of lymphocytes derived from the epithelium. The cytokine production was evaluated by ELISA assays and the results were reported for 1×10^6 cells. Each point represents a biopsy. Exo and TZ = normal exocervix and transformation zone biopsies of patients with normal cervix; EXOs and SIL = normal exocervix and SIL biopsies of patients with SIL; ND = undetermined.

