Dynamics of cervical Langerhans cell counts in the course of HPV-positive CIN treatment with the use of human recombinant interferon gamma

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Summary

Objectives: Langerhans cells play a pivotal role as professional antigens presenting cells in cervical epithelium, thus changes in their density or/and function may profoundly influence the proper activation of the afferent arm of immune response in cases of HPV-related intraepithelial lesions.

Aim of the study: Assessment of intraepithelial Langerhans cell count changes in CIN I/CIN II after human recombinant interferon gamma (IFNγ) application and correlation with clinical outcome.

Material & Methods: The present study is a part of prospective trial on IFNγ application in the treatment of CIN I/CINII associated with high-risk HPV infection. Seventeen subjects underwent uniform IFNγ treatment (four intracervical injections in a two-day interval for a total dose of 6,000,000 IU). Langerhans cells were stained within cervical punch biopsy specimens with the use of polyclonal anti-S-100 antibody according to the three-step indirect peroxidase protocol, and their mean count calculated for the following groups: before IFNγ treatment launching, immediately after completion of the treatment, and after two months of follow-up.

Results: The analysis revealed a rapid and significant increase in Lagerhans' cell count immediately after treatment completion (21.17/mm^2 and 25.94/mm^2, respectively, at p = 0.019) which further increased in the group of complete response (in 9 subjects; 32.22/mm^2). After transient elevation of the Langerhans' cell count it returned to a level even lower than initially in the non-responder group (4 subjects; 20.25/mm^2).

Conclusion: Our data strongly support the observation from static studies suggesting that regression of HPV-related cervical lesions is associated with increased density of epithelial Langerhans cells.

Key words: Langerhans cells; Cervical intraepithelial neoplasia; Interferon gamma.

Introduction

Initially described in 1868, Langerhans cells are now considered to be a population of bone marrow-derived dendritic cells migrated to epithelia and skin, where they constitute a small proportion (3% to 8%) of epithelial cells [1]. It has been proven that they play a sentinel role in the processing and presenting of exogenous antigens as well as in specific T-cell activation, being a part of mucosal associated lymphoid tissue (MALT) [2], with the preferential location around the external cervical os [3]. Several surface markers of Langerhans cells have been determined (Fc IgG and C3b complement component receptors, S-100 protein, OKT 6, and HLA-DR), and it is supposed that at least S-100 positive cells constitute their distinct subpopulation with individual behaviours [4].

Many studies have addressed the question of Langerhans cell density in HPV-induced cervical lesions, including cervical cancer, yielding inconsistent results. The profound depletion of Langerhans cells in HPV-associated cervical intraepithelial neoplasia and HPV-productive infection in comparison to lesion-free epithelium has been demonstrated [1, 4-6]. It is therefore speculated that human papillomavirus infection, by reducing intraepithelial Langerhans' cell counts, may negatively affect local immune afferent limbs and thus have a promoting influence on the development of higher grades of cervical lesions, including cancer. On the other hand, however, once intraepithelial HPV-dependent lesions are established the density of epithelial Langerhans cells in these regions increases along with grade of CIN, which may reflect a specific immune response directed against neoantigens associated with malignant transformation [6]. A significant drop in Langerhans' cell counts is observed in cases of invasive cervical cancer [7, 8] which a tempts to suggest ineffective immune surveillance in these circumstances. Contradictory to this are reports showing a gradual but significant decrease in Langerhans cell counts along with increasing CIN grade [9-12], probably statically selecting the cases with no adequate immune response.

The shortage or even lack of prospective follow-up studies on the dynamics of Langerhans' cell counts in the course of HPV-related lesions prompted us to undertake the current study. The primary objective of the research was to trace the changes of epithelial Langerhans' cell density in individual cases of CIN during conservative treatment with intracervical applications of human

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recombinant interferon gamma. We aimed to study the relation among the Langerhans' cell counts and CIN regression/persistence states.

Material and Methods

Immunohistochemical assessment had been carried out on the samples of cervical punch biopsy specimens taken during the clinical prospective trial on the efficacy of human recombinant interferon gamma 1-b in the treatment of cervical intraepithelial neoplasia, from August 1995 to October 1997 (INTERCIN trial). The University School of Medicine in Lublin Ethical Board approval of the described trial was obtained twice: at the beginning and at the completion of it. All the enrolled patients had given written consent for the procedure under study.

Patient characteristics

The group of women enrolled in the study comprised 17 patients fulfilling the following criteria: (1) cervical intraepithelial neoplasia (CIN) grade I or II established on the basis of initial pathological examination of colposcopically guided cervical punch biopsy (2) no spontaneous regression of CIN during the four-month observation period (i.e. between the initial diagnosis and implementation of IFNγ treatment) (3) presence of DNA HPV high-risk as detected by the Hybrid Capture System (4) written consent given for the therapy and follow-up (5) pregnancy excluded on the basis of negative β-hCG assessment.

The mean age of the patients was 37.4 (range 24-45); three patients were nulliparous and the remaining 14 had given birth one to five times. Five patients had previously undergone electrocoagulation of the cervical portio due to glandular ectopy and in one case cold knife conisation had been performed due to a low-grade squamous intraepithelial lesion. There were only two cases of caesarean sections in the obstetric history of two patients.

Interferon gamma treatment schedule

All the subjects underwent a uniform protocol of interferon gamma treatment. IFNγ 1-b (Imukin, *Boehringer Ingelheim* GmbH, Ingelheim am Rhein, Germany) at a single dose of 1,500,000 IU (0.05 mg) was injected intracervically at the periphery of the lesion in the central part and close to the external cervical os under colposcopic guidance with the use of a 2 ml syringe and 0.6 mm needle. Injections were repeated four times in two-day intervals, to a total dose of 6,000,000 IU (0.2 mg) of IFNγ. Treatment was launched in each case in the early proliferative phase of the menstrual cycle and carried out according to hospital protocol.

HPV-DNA detection

Detection of DNA HPV was performed using the commercially available Hybrid Capture System (Digene Diagnostic, Silver Springs, Co. USA) in cervical smear specimens. Identified high-risk HPV types included (RNA probes cocktail): 16/18/31/33/35/45/51/52/56. HPV-DNA detection was carried out twice: at the beginning of the treatment and two months after completion.

Tissue samples

Cervical tissue samples were obtained by colposcopically guided punch biopsy using Tischler's biopsy forceps (*Aesculap,* Tutlingen, Germany) from the site of atypical epithelium (secondary aceto-white appearance, iodine negative, and/or reversed mosaic or punctuaction). The diameter of tissue sampled did not exceed 2.5 mm, and in no case caused total excision of the lesion. The average proportion of a biopsied lesion was 18%, therefore there was no case of a lesion which could be biopsied away. In cases of morphological regression the biopsy site was determined on the basis of previous colpophotographical documentation. Local anesthetics were not needed. Immediately after obtaining tissue samples they were placed in buffered 9% formalin solution followed by paraffin embedding.

Timing of cervical sampling

The first punch biopsies were taken 14 to 21 days (average 18 days) prior to treatment launching (first interferon gamma injection) in the early or mid proliferative phase of menstrual cycle - group A; the next biopsies were carried out three days after the last interferon gamma injection - group B; and, the last set of biopsies: two months after treatment completion - group C. The latter group was further divided into three subgroups on the basis of pathological findings and HPV-DNA typing: C - CR (complete response): with no morphological signs of intraepithelial lesions and negative result of HPV-DNA detection; C - PR (partial response) - with either lower grade of CIN compared to initial or/and negative HPV-DNA detection; and C - NR - (no response) with intraepithelial lesions of the same grade as initially and presence of HPV-DNA.

Treatment outcome measures

The results of treatment were assessed two months after treatment completion on the basis of punch biopsy pathologic examination and HPV-DNA typing. Cases with no histopathological features of cervical intraepithelial neoplasia and no high-risk HPV-DNA were regarded as complete response. The cases where a lower grade of CIN was noticed (i.e. CIN I instead of CIN II) or/and negative for high-risk HPV DNA were regarded as partial response.

Immunohistochemical staining

An indirect immunoperoxidase staining technique was applied using the avidin-biotin protocol.

Paraffin embedded sections were cut at 4-6 (m thickness, mounted on precoated glass slides (S3003; *DAKO,* Copenhagen, Denmark) and air-dried at 37°C for 24 hours. The sections were deparaffinized in xylene and graded alcohol series. To inactivate endogenous peroxidase, sections were incubated in 3% H_2O_2 for five minutes. Subsequently sections were incubated with the primary antibody: rabbit anti-cow S-100 polyclonal antibodies (DAKOPATTS a/s Z311, *DAKO,* Copenhagen, Denmark) in 1:100 dilution using PBS/BSA (phosphate buffer saline/bovine serum albumin) as a solvent for 45 min. The next stage of the procedure was the incubation of slides with the diluted (1/35) biotinylated secondary antibody - swine anti-rabbit immunoglobulins (DAKO-immunoglobulins a/s P217) for 45 minutes. In the last stage they were incubated with the avidin and biotinylated peroxidase complex (Super ABC Kit, NCL-ABCu; *Novocastra*, Newcastle upon Tyne, UK). After each stage sections were washed three times with 0.05 M TRIS-HCL buffer. Peroxidase activity was detected with DAB (diaminobenzidine tetrahydrochloride). All incubation steps were performed at room temperature. Negative controls consisted of parallel sections incubated with buffered solution of rabbit IgG2 and omission of the primary antibody. Slides were lightly counterstained with haematoxylin.

Langerhans' cell counts

The quantitative assessment was performed with the use of a Nikon Eclipse E 200 microscope (*Nikon Corp.,* Tokyo, Japan) at a magnification of 250x combined with a computer image

analysis system. The system was composed of a colour camera, PC computer with a Pentium III processor and frame grabber card, colour monitor 17" and software for Windows LUCIA G (M) version 4.11 (Laboratory Imaging Ltd., *Precoptic Co.*, Warsaw, Poland). After calibrating the system the number of Langerhans cells on 1 mm^2 were calculated as the mean from counting of five pools of 1 mm^2 on each slide examined.

Statistical analysis

The mean number of Langerhans cells were calculated for each group of specimens and the differences among them assessed with the application of the Mann-Whitney-U test. Differences were considered significant at $p < 0.05$.

Results

Clinical outcome

Two months after treatment completion on the basis of the established criteria, complete regression (absence of epithelial lesions and negative high-risk HPV DNA testing result) was obtained in nine subjects (52.9%). Partial response was noted in four cases (23.5%): in two cases lower grade of CIN, in three cases no high-risk HPV DNA (one case comprised both lower grade of CIN and HPV DNA negative result). The total proportion of responses obtained (complete and partial) in the study was 76.5%. A detailed analysis of the clinical outcome, side-effects and follow-up is described elsewhere [13-16].

Dynamics of Langerhans' cells count

Figures 1 and 2 show the examples of S-100 (+) Langerhans cells in dysplastic epithelium before treatment launching and immediately after completion. A slight but significant increase in mean epithelial Langerhans' cell counts was noticed immediately after the full dose of IFNγ application (21.17/mm^2 vs 25.94/mm^2, $p = 0.019$). A further significant increase in Langerhans cell counts appeared two months after treatment completion only in the group with complete response (32.28/mm^2) in comparison to the initial count ($p < 0.005$) or immediate post-treatment count ($p = 0.025$) (Tables 1 and 2).

In the group where only partial response occurred there was a further slight but not significant increase in Langerhans' cell counts two months after treatment compared to the immediate post-treatment count (25.94/mm^2 vs 27.00/mm^2, $p = 0.76$). Comparing final mean Langerhans' cell counts between the group with complete

Table 1. — *S-100 positive Langerhans' cell counts in the individual groups of biopsy specimens.*

Group of biopsy samples			Statistical parameters	
Group	No. of slides	Mean Langerhans cell counts in 1 mm^2	Standard deviation	Standard error
A	17	21.17	4.37	1.06
B	17	25.94	6.68	1.62
C – CR	9	32.22	5.78	1.92
C – PR	4	27.00	2.94	1.47
C – NR	4	20.25	3.86	1.93

Table 2. — *Results of statistical analysis of Langerhans' cell count differences between individual pairs of biopsy samples groups.*

Difference between groups		Value of subtraction	p value	Statistical significance
A vs:	B	- 4.77	0.019	S
	C - CR	- 11.05	< 0,005	S
	C - PR	- 5.83	0.02	S
	C - NR	0.92	0.7	NS
B vs:	C - CR	- 6.28	0.025	S
	C - PR	- 1.06	0.76	NS
	C - NR	5.69	0.12	NS
C – CR vs:	C - PR	5.22	0.12	NS
	C - NR	11.97	0.003	S
C – PR vs:	C - NR	6.75	0.03	S

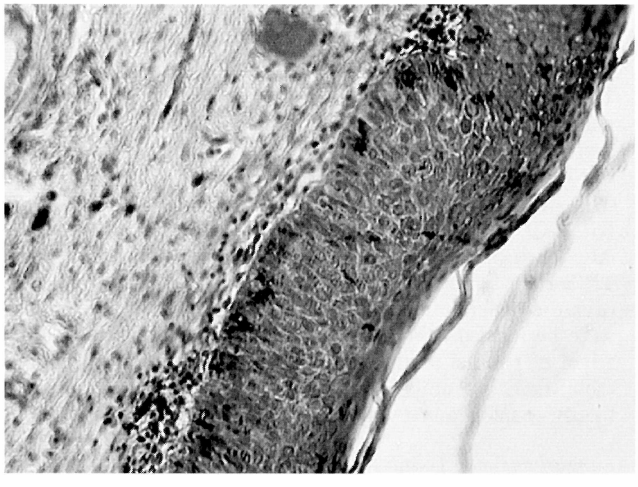

Fig. 1

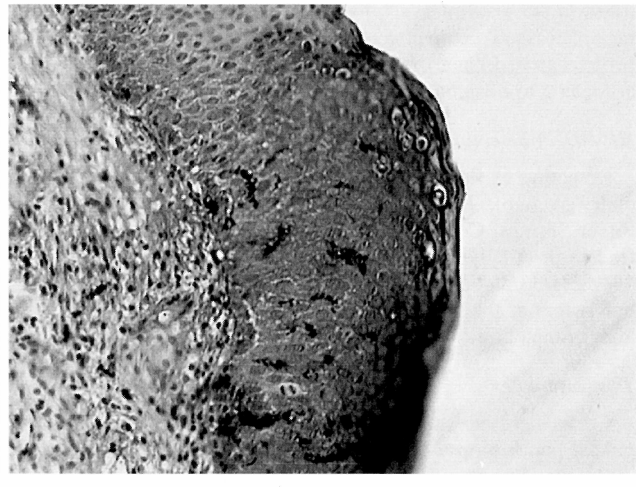

Fig.

Figure 1. — Example of S-100 positive Langerhans' cell density and intraepithelial distribution before treatment launching.
Figure 2. — Example of S-100 positive Langerhans' cell density and intraepithelial distribution immediately after treatment completion.

regression and partial response the difference was not significant ($p = 0.12$), however a trend toward a higher count in the group with complete regression was seen. In four cases of non-responders the final Langerhans' cell counts showed a minor but not significant depletion in comparison to the initial count (20.25/mm^2, $p = 0.7$), and was significantly lower compared to the complete regression group ($p = 0.003$) and the partial regression group ($p = 0.03$) (Tables 1 and 2).

Discussion

Direct analysis of the obtained results indicates that intracervical application of human recombinant interferon gamma, even in moderate doses, brings a significant and rapid increase in mean intraepithelial Langerhans cell (LC) counts. Such an effect can be partly ascribed to a potent IFNγ influence on keratinocyte granulocyte-macrophage colony stimulating factor (GM/CSF) production [17]. GM/CSF is a well known cytokine, essential for promoting dendritic cell differentiation [18, 19]. Hubert *et al.* recently described its role in the colonization of in vitro-formed cervical human papillomavirus-associated preneoplastic lesions with dendritic cells [20]. In their model HPV-transformed keratinocytes contained low amounts of GM-CSF and induced only a weak motile response of LCs, which could have been greatly improved by adding recombinant GM-CSF to the media. The neoplastic epithelial profile of cytokines produced by HPV-transformed keratinocytes may be altered or not sufficient. However in our model, comprising only cases of cervical intraepithelial neoplasia up to CIN grade II, a considerable proportion of epithelium was not transformed, being therefore sensitive to IFNγ stimulation, and as theoretically expected, vulnerable to increased GM-CSF production.

Another potent activator of Langerhans cells - tumor necrosis factor (TNF α) - was expressed constitutively by basal keratinocytes of normal cervix, but expression of this cytokine was greatly diminished in the majority of CIN cases studied by Mota *et al.* [21]. Conversely, the suppressive cytokine, Il-10, was absent in normal epithelium but up-regulated in half of the studied CIN cases. Both *in vivo* and *in vitro* studies show that interferon gamma shifts the balance of Th lymphocyte cytokine production to the Th1 type, which includes increased production of IL-2, TNF-β and GM-CSF, while decreasing Th2-type cytokine panels, with IL-10 among them. These properties of IFNγ contribute to the increase of LC number demonstrated in the study, and, which is very likely, also to Langerhans cell activity and antigen expression.

The striking finding in our study was further increase in LC counts two months after treatment completion noticed in the regressor cases, with only a moderate and non-significant increase in partial regression cases, and even a drop down in the non-regression group. It seems that initial changes induced by interferon gamma application tend to increase in cases predestined to regression. We are not able to make any direct comparisons to the literature data, as the presented dynamic study is unique in its design. The final mean LC count in our study in the group with complete regression (secondary metaplastic squamous epithelium) reached 32.22/mm^2, being consistent with previous reports on LC density in normal cervical epithelium [4, 9].

Results of our dynamic study support the suggestion that regression of cervical intraepithelial lesions is associated with increased LC density, which comes from few previous static studies. Fukuda *et al.* demonstrated a significantly higher LC count in cases of regressing cervical dysplasia than in cases of persistent lesions (15.1/mm^2 vs 8.6/mm^2 respectively, $p < 0.0002$) [22]. Recent extensive static analysis by Connor *et al.* revealed significant depletion of S-100 positive LCs in cases of low- and high-grade intracervical lesions compared to normal epithelium (8.6 and 6.0 /mm vs 16.7/mm, respectively) as well as in cases with HPV infection (5.9 vs 12.8/mm in non-infected cases) [9].

In relation to advanced cervical cancer it has been noted that rates of 5-year survival have been significantly better for patients with Stage III tumours infiltrated by LCs, compared to the group without such infiltrates [23]. Also improved results of radiotherapy of advanced adenocarcinoma of the cervix have been achieved in cases with LC infiltrates, particularly with sizofiran modulation [24, 25].

Decreased Langerhans cells have been reported in relation to HPV type infecting the epithelium, however observations in this field do not seem to be conclusive. Similar depletion of LCs was associated with HPV6 & 11 (classically not related to malignancy) as with HPV16 & 18 as observed by Morelli *et al.* [6]. Hawthorn *et al.* reported an apparent relation between decreased Langerhans cells and moderate to high HPV16 DNA load, with a significant reduction of LC count more pronounced in cases of HPV18 infection even at low copy numbers [26]. Similar observations came from study by Lehtinen *et al.* [27], which suggest that the depletion of LCs is associated with productive HPV16/18 infection of the cervical epithelium. The Hybrid Capture System applied in our study did not allow us to determine the persistence of low-risk HPV types, nor to determine the correct sequelle: HPV clearance following increased LC counts or the opposite. To answer this question more frequent DNA HPV testing or *in situ* PCR techniques might provide some help.

The mechanism in which Langerhans cells contribute to resolution of HPV-transformed keratinocytes is certainly complex. Observations of merely their numbers do not provide the insight into their functional state. Langerhans cells as the immature dendritic cells located in the peripheral tissue capture and process antigens. There is a wide range of stimuli causing induction of dendritic cell maturation, among which viruses and certain cytokines are very potent. Maturation of dendritic cells is associated with up-regulation of co-stimulatory molecules and antigens coded by major histocompatibility complex (MHC). Mota *et al.* observed a significant upregulation

of HLA-DQ by Langerhans cells in high-grade intraepithelial cervical lesions (very weak expression of this antigen in normal cervices) suggesting antigen-presenting cell activation in papillomavirus-related premalignant disease [28]. In experimental conditions acute activation of dendritic cells could have been elicited by exposing them to HPV 16 virus-like particles (VLP), however the addition of syngenic T cells or recombinant interferon gamma was required [29]. After antigen acquisition Langerhans' cells present it complexed to MHC molecules and activate T cells, which may lead to lysis of antigen-bearing cells or antibody production.

Our observations concerning Langerhans' cell morphology confirm previous reports indicating more prominent dendritic aspects of cells in the superficial layers of epithelium [30]. We could not, however, definitely confirm the clustering of the LCs near capillaries and lymphocyte aggregates or focal lymphocytic infiltrates which, according to Tay *et al.*, might have been the sign of LC involvement in the process of antigen presentation and T cell activation [4]. Apart from more even distribution of LCs throughout the entire epithelial thickness in cases with increased LC counts after completion of IFNγ treatment no other histopathological features could be found.

Our prospective study comprised a relatively limited number of consecutive cases, but its strength comes from consistent diagnoses of cervical intraepithelial lesions and universal therapy protocols thus enabling us to follow the dynamics of Langerhans cells in regressing and persisting lesions.

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