

Cytokine serum levels in patients with cervical intraepithelial neoplasia grade II-III treated with intralesional interferon- α 2b

Daniela Ribeiro Misson¹, Douglas Reis Abdalla², Ariana Melo Borges², Denis Sakamoto Shimba³, Sheila Jorge Adad⁴, Márcia Antoniazzi Michelin⁵, and Eddie Fernando Candido Murta⁶

¹Gynecology and Obstetrics, and ²General Pathology, Oncology Research Institute (Instituto de Pesquisa em Oncologia-IPON); ³Oncology Research Institute (IPON)/Discipline of Gynecology and Obstetrics; ⁴Discipline of Special Pathology; ⁵Oncology Research Institute (IPON)/Discipline of Immunology; ⁶Oncology Research Institute (IPON)/Discipline of Gynecology and Obstetrics, Federal University of the Triângulo Mineiro, Uberaba, Minas Gerais, Brazil

ABSTRACT

Aims and background. Cervical intraepithelial neoplasia (CIN) grade II-III is being diagnosed in younger women and, because of the reproductive age range for women and the habits associated with a modern lifestyle, is now affecting a broad age range. Surgical treatment for CIN has been associated with premature amenorrhea, low birth weight, and premature labor and birth. It is therefore imperative to develop clinical treatments for CIN, such as conservative treatment with interferons. The object of the present study was to evaluate the behavior of cytokines (IFN- γ , IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, TNF- α , TGF β) in the serum of patients with an initial diagnosis of CIN II-III.

Methods. Ten patients with CIN-CIN II (60%, n = 6) and CIN III (40%, n = 4), 23 to 51 years of age and who had not received any prior treatments, were evaluated. The patients were given 3 million/UI (per cm² of colposcopic lesion) of human recombinant IFN- α 2b by intralesional administration (18 applications on alternate days). Before treatment, in the 6th, 12th, and 18th applications, blood was collected from the patients for cytokine analysis using ELISA.

Results. Half of the patients had a good pathologic response; the other half, all of whom were smokers, had therapeutic failure. The average concentration of IL-12 (pg/ml) in the serum of patients who responded well to therapy was elevated from the 12th and 18th application of IFN- α 2b compared to patients who experienced therapeutic failure: 1804.0 \pm 1020 vs 391.2 \pm 722.3 and 1738.0 \pm 2426.0 vs 448.5 \pm 407.2, respectively, $P < 0.05$.

Conclusions. CIN II-III treated with intralesional IFN- α 2b achieved a good response in non-smoking patients and was associated with an increase in IL-12 serum levels.

Introduction

Cervical intraepithelial neoplasia (CIN) is a common neoplastic change affecting women in a reproductive age. Low-grade CIN can resolve without treatment or develop into cervical cancer¹, the second most common cancer among women worldwide². In Europe, the incidence and mortality rates for cervical cancer are substantially higher in Eastern Europe than in Western Europe, mainly because of the lack of effective prevention³. Recognition of the key role of high-risk forms of human papillomavirus (HPV) in the etiology of cervical cancer has led to the development of prophylactic vaccines and the incorporation of HPV tests in triage. In the European Union, HPV testing is an accepted part of the triage when cytology results are uncertain and of tracking after treatment for CIN II-III⁴.

Key words: cervical intraepithelial neoplasia II-III, cytokines, interferon α -2b, interleukin-12.

Acknowledgments: The authors would like to thank the Studies and Projects Funding Body (Financiadora de Estudos e Projetos, FINEP), the Foundation for Research Assistance of the State of Minas Gerais (Fundação de Amparo à Pesquisa do Estado de Minas Gerais, FAPEMIG), the National Council for Scientific and Technical Development (Conselho Nacional de Desenvolvimento Científico e Tecnológico, CNPq), and the Uberaba Foundation for Teaching and Research (Fundação de Ensino e Pesquisa de Uberaba, FUNEPU) for financial assistance.

Correspondence to: Eddie Fernando Candido Murta, MD, PhD, Oncology Research Institute (IPON)/Discipline of Gynecology and Obstetrics, Federal University of the Triângulo Mineiro (UFMTM), Avenida Getúlio Guaritá, s/n° Uberaba (MG), Brazil, CEP 38025-440, Bairro Abadia. Tel +55-34-3318-5326; fax +55-34-3318-5342; e-mail eddiemurta@mednet.com.br or eddiemurta@pq.cnpq.br

Received January 27, 2011; accepted April 8, 2011.

Despite the implementation of prophylactic measures, high-grade cervical lesions are being found in younger and younger patients, who are, in most cases, nulliparous or primiparous. The therapies most often used are cold knife conization and the loop excisional electric procedure. These are invasive, and consequently relatively aggressive treatments which increase the risk of premature labor, premature amniorrhexis, and low birth weight⁵⁻⁷. Therefore, new studies of less invasive treatments, such as immunotherapy, are needed. Proteins from the interferon (IFN) family have been used in the clinical treatment of cancer, with varying but promising results, with IFN- α being the most widely used cytokine⁸. Studies have shown remission in lesion size in 30-80% of CIN cases following treatment with IFN- α ⁹⁻¹¹. With respect to invasive neoplasia, cases of invasive vaginal carcinoma being cured by intralesional IFN- α 2b treatment have been reported¹².

The anti-tumor properties of IFN- α result from its direct action on viral proliferation and/or induction of the inhibition of growth factors specific to the cells. IFNs can also have indirect effects, such as immunomodulation and inhibition of tumor angiogenesis⁹. Our group has observed elevated expression of Th1 molecules [IFN- γ , tumor necrosis factor (TNF)- α , and IL-2], with a significant reduction of high-risk HPV viral load, in the cervical stroma of patients treated with IFN- α 2b who obtain a good therapeutic response¹³.

Cytokines are known to be liberated during immune response, acting in the regression or persistence of lesions associated with HPV. Understanding the immunological modifications that occur during IFN therapy will be important to develop strategies to treat cancer and precancerous lesions. Thus, in order to better understand immunological responses in patients with CIN II-III, the objective of this study was to analyze the concentration of cytokines (IFN- γ , IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, TNF- α , TGF- β) in patients' serum, before and after treatment with intralesional IFN- α 2b.

Materials and methods

Setting and patients

A prospective study was performed at the Maria da Glória outpatient clinic of the Hospital School of the *Federal University of the Triângulo Mineiro*, in the departments of Gynecology and Immunology from 2007 through 2009. The group studied consisted of 10 patients between 23 to 51 years of age, with diagnoses of CIN II-III, who had not received any prior treatment. Patients provided information about their ages, habits, lifestyles (e.g., smoking, use of drugs, number of partners), contraceptive methods used, history of sexually transmitted diseases, and use of hormone replacement therapy (Table 1). All procedures performed followed the criteria developed

by the Ethics Committee (CEP/UFTM Nos. 759 and 1525). The inclusion criteria were: absence of bleeding during the examination; no use of oral antibiotics, vaginal fungicides or creams over the previous 30 days; no sexual activity for two days preceding sample collection; no previous history of treatment for HPV; and no colposcopic change <1 cm². The exclusion criteria were: immunosuppressant diseases, serious cardiopathies, changes in liver or kidney function, pregnancy, a reported intolerance to IFN, or an absence of a visible lesion at colposcopy.

Application of IFN

Human recombinant IFN- α 2b (alpha IFN 2b-Blaferon-B[®]; 10,000,000 U) was used for the therapy. The IFN- α 2b was applied intralesionally at a dose of 3,000,000 UI (flask-ampoule with lyophilic powder diluted in 1.0 ml of diluent before each application). The applications were performed using a 1.0-ml syringe with a 13 \times 0.45 needle three times a week on alternate days until a total of 18 applications were reached.

Exposure of the cervix was performed by introduction of a vaginal speculum. The medication was applied following antiseptics of the cervix and the vaginal walls with gauze soaked in topical povidone using Sheron forceps. After the first, sixth, twelfth, and eighteenth application of IFN- α 2b, peripheral blood was collected from the vein of the right forearm of each patient. Serum was separated out and stored at -80 °C for cytokine evaluation.

Evaluation of clinical response

The groups evaluated in the study were based on colposcopic examination, biopsy and histology. Thus, if colposcopy showed disappearance or regression of the lesion, as confirmed by histological examination from biopsy, with regression to CIN I or no CIN, the treatment was considered as satisfactory or successful, characterizing the good response group (GR). The patients were submitted to follow-up with cytology and colposcopy every 6 months.

If no regression of the lesion was observed at colposcopic examination, confirming the persistence of CIN II or III in biopsies, failure of the treatment was considered, characterizing the bad response group (BR). All patients with CIN II and III were immediately submitted to cold knife conization.

Cytokine levels

The presence of cytokines (IFN- γ , IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, TNF- α and TGF- β) in the patients' serum was measured by enzyme linked immunosorbent assay (ELISA) using pairs of monoclonal antibodies purchased from BD OptEIA[™] (BD Biosciences, San Diego, CA, USA). The procedure was performed in accord with the manufacturer's protocol. Plates with 384 wells were sensitized with 25 μ l of specific monoclonal antibodies for uptake of the desired cytokine diluted in a

Table 1 - Clinical characteristics, histological diagnosis by biopsy, and conduct in each case, after IFN treatment

Patient	Age	Smoker	P/D/M	Contraception	First intercourse (yr)	First pregnancy (yr)	Initial diagnosis	Final diagnosis	IFN treatment outcome
1	36	No	1/1/0	Oral	WN	WN	CIN III	Benign cellular changes	Response
2	51	No	5/3/2	WN	21	23	CIN II	CIN I	Response
3	30	No	0/0/0	Oral	17	WN	CIN II	Chronic inflammation	Response
4	25	No	4/3/1	ID	14	15	CIN II	Normal	Response
5	23	No	0/0/0	Oral	15	WN	CIN III	CIN I	Response
6	49	Yes	2/2/0	Oral	WN	WN	CIN II	CIN III	Failure
7	30	Yes	1/1/0	Oral	15	22	CIN III	CIN II	Failure
8	35	No	0/0/0	WN	WN	WN	CIN II	CIN II/VAIN III	Failure
9	46	Yes	6/5/1	Oral	13	18	CIN III	CIN III	Failure
10	32	Yes	1/1/0	Condom	14	18	CIN II	CIN II	Failure

P/D/M, Pregnancy/Delivery/Miscarried; WN, without notification; ID, intrauterine device.

coating buffer. For the standards, we added 25 μ l of recombinant cytokine to wells in the first row of each plate according to a 1:2 dilution series in assays diluted based on the initial concentrations indicated. To the other rows, 25 μ l/well of patients' serum containing the cytokine to be dosed was added. The plates were incubated at room temperature for 2 h and washed five times with a solution containing 20% PBS-Tween. Then, 25 μ l/well of detector antibody for the cytokine to be dosed was added. The plates were incubated for 1 h at room temperature and washed again five times in PBS-Tween. After this step, 25 μ l/well of TMB Substrate Reagent Set (BD OptEIA™) was added, and after 30 min, 25 μ l/well of Stop Solution (2N phosphoric acid) was added. After adding the Stop Solution, a reading of the ELISA plate was performed using the Spectra^{max} 384 Plus automatic reader, with the results being obtained by the difference between the 450-nm and 570-nm absorbances. The concentrations of each of the patients' serum cytokines were expressed in pg/ml through comparison of the absorbances obtained by the standard curve of the respective cytokine, which was obtained simultaneously.

The sensitivity of the cytokines assayed is: 4.7-300 pg/ml for IFN- γ , 3.9-250 pg/ml for IL-1 β , 15.6-1000 pg/ml for IL-2, 7.8-500 pg/ml for IL-4, 4.7-300 pg/ml for IL-6, 3.1-200 pg/ml for IL-8, 7.8-500 pg/ml for IL-10, 31.3-2000 pg/ml for IL-12, 7.8-500 pg/ml for TNF- α and 125-8000 pg/ml for TGF- β .

Statistical analysis

An electronic data base was developed for the statistical analysis. The variables were analyzed using the GraphPad Prism 4.0 program. The values were submitted to Student's *t* test. The differences were considered statistically significant when $P \leq 0.05$.

Results

We examined the blood of 10 patients with CIN II-III who were treated with IFN- α 2b (18 applications, with

blood collected from the patients at the first, sixth, twelfth and eighteenth application). Of the 10 patients treated with IFN- α 2b, 50% (5/10) achieved a good clinical response (CIN II or III regression to CIN I or CIN absent as confirmed by biopsy); these patients constituted the good responder (GR) group. The remaining 50% (5/10) did not respond to the treatment (persistence of CIN II or II on biopsy) and constituted the bad responder (BR) group. The patients in the GR group ranged in age from 23 to 51 years and in the BR group from 30 to 49 years. In the GR group, 4/5 patients achieved a complete response and the remaining patient achieved a partial response, with the lesion going from high grade to low grade. Interestingly, only one patient who did not respond to the IFN- α 2b treatment was a non-smoker.

Distinct patterns were observed in the blood cytokine data between the GR and BR groups. The concentrations of these cytokines are shown in Table 2. The Th1 cytokine pattern showed great oscillation in the concentrations of cytokines in the GR patients, with the IFN- γ and TNF- α cytokines decreasing and IL-2 and IL-12 increasing over the course of IFN- α 2b treatment. There was a significant increase in IL-12 for the BR group on the 12th and 18th applications (Figure 1). In general, the BR patients did not show statistically significant changes in the synthesis of these cytokines.

Our analysis of the Th2 and Treg cytokine pattern did not reveal discernable levels of IL-4. IL-10 exhibited patterns of expression similar to the other groups, although it had diminished expression on the second application in the GR. Over the course of the IFN- α 2b treatment, the GR group had stable TGF- β levels whereas the BR group showed a general decrease in TGF- β levels. TGF- β levels in BR patients remained higher than those in GR patients at all times (data not showed).

Analyses of IL-1 β , IL-6, and IL-8 revealed IL-1 β oscillations in both groups. Levels of the IL-6 cytokine decreased over the course of IFN- α -2b therapy in the GR group, IL-8 showed no changes in the GR group, but appeared to decrease in the BR group. The final IL-8 levels

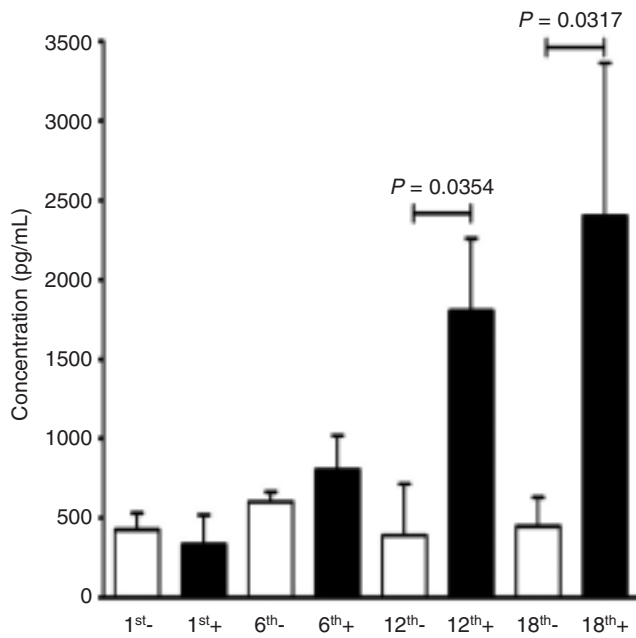


Figure 1 - Behavior of IL-12 in GR group (black) versus BR group (white) over the course of therapy. GR, good response; BR, bad response.

in the GR group remained higher than in the BR group (data not shown).

Figure 2 shows the IL-12 level of each patient according to clinical response. Patients with clinical regression (GR group) produced much more IL-12 than those without clinical regression of lesions.

Discussion

Few data have been published on the expression of cytokines in the serum of patients with CIN II-III being treated with intralesional IFN- α 2b. Through our evaluation using the ELISA method, we observed that during

the treatment period, Th1 profile cytokines were more highly expressed in patients who achieved a GR than in those with a BR.

Cervical carcinoma can have well-defined pre-invasive stages, together with viral factors involved at the molecular level, which makes it a good model for investigating alternative treatments using immunotherapies¹⁴⁻¹⁶. Thus, our study examined the influence of immunological therapy for CIN II-III on cytokine behavior during intralesional IFN- α 2b treatment, through analysis of peripheral serum of patients.

Factors that increase the risk of HPV infection and the development of CIN include smoking, parity¹⁷, the number of partners a woman has had, and an early sexarche¹⁸. The diagnosis of high-grade lesions often occurs in women between 30 and 45 years of age^{19,20}.

The contribution of smoking to cervical oncogenesis presumably stems from direct exposure of the DNA of cervical epithelial cells to cigarette compounds, such as nicotine and cotinine, which are present in high concentrations in the cervical mucus, where they can promote mutation and changes in genetic activity²¹. Smoking causes changes not only in the tissues, but also in the immunological system; it increases T suppressor lymphocyte levels, decreases natural killer cell levels, and decreases immunoglobulin levels. In addition, smoking suppresses the chemotactic and phagocytic functions of the polymorphonuclear cells in tissues, thereby increasing susceptibility to pathogens^{13,22,23}. The present observations reinforce this point as most of the patients who achieved a poor therapeutic response with IFN- α 2b were smokers, whereas most of the patients who obtained a good response were not smokers.

Studies examining IFN therapy have shown good responses with IFN- α , IFN- β , and IFN- γ , with results ranging from total and partial regression of cervical lesions to an immune modulation favorable to the patient^{10,12,24-30}. These prior data were corroborated by the present study, as 4/5 of the patients treated with IFN- α 2b that obtained a good response were non-smokers,

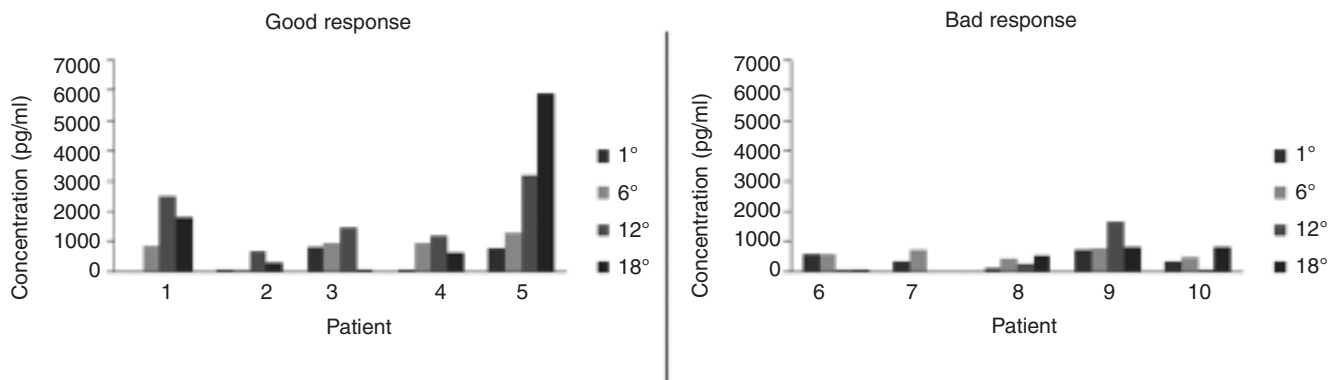


Figure 2 - Measurement of serum IL-12 of each patient with good response (GR) or bad response (BR). 1st, 6th, 12th and 18th application.

whereas all 5 patients that had a bad response were smokers.

A study by Rensing *et al.*³¹ reported that the absence of IL-2 mRNA in lesions caused by HPV can be explained by the weak response of memory TCD8⁺ lymphocytes in patients with CIN and cervical carcinoma. There is also evidence indicating that circulating lymphocytes produce IL-2 molecules specifically in response to HPV antigens³².

However, the presence of TNF- α promotes progression of the cell cycle by increasing HPV-16 E6/E7 mRNA expression, which functions to immortalize HPV-infected keratinocytes³³. We observed a lower concentration of TNF- α in the GR group than in the BR group.

An association between the expression of IL-12 mRNA *in situ* and regression of CIN III lesions has been reported, suggesting that the Th1 immune response mediated by IL-12 may be related to overcoming HPV infection³⁴. Our findings strongly reinforce this possibility, as the concentration of IL-12 at the systemic level was significantly higher in the GR group than in the BR group. This increase may be related to the action of dendritic cells, which produce IL-12 and require the presence of IL-1 β above a certain level³⁵. Indeed, the present finding that the GR group also expressed higher concentrations of IL-1 β than the BR group provides further support for this view.

In contrast to our findings with Th1 cytokines, we found lower levels of cytokines of the Th2 and Treg profiles in patients with a good response, relative to those with a poor response. The patients who did not respond to treatment mainly showed increases in Th2 cytokine levels. Hence, increases in the production of Th2 cytokines, such as IL-4 and IL-10, may be triggered by tumor cells as a mechanism to help them evade immune recognition³⁶. Indeed, increased levels of these cytokines have been associated with the persistence and progression of pre-malignant lesions^{37,38}.

A study by Ramos *et al.*¹³ was not able to relate the expression of TGF- β mRNA with clinical response to treatment with IFN- α 2b, because the patients who received a positive response expressed TGF- β , which was not expressed by patients with treatment failure. However, in the present study, we found that patients in the GR group showed constant levels of TGF- β , whereas patients of the BR group had higher levels of TGF- β than those of the GR group. Thus, it is suggested that the combined action of Th1 overlaps the isolated action of TGF- β in the CIN.

Likewise, we observed higher levels of IL-6 and IL-8 in the BR group, a finding which corroborates similar results in vaginal secretions³⁹. Elevated levels of IL-6 and IL-8 have been implicated in the promotion of tumor angiogenesis and the development of cervical cancer^{40,41}.

Analyzing the data in Table 2, the non-responding patients apparently present lower levels of IL-1 β and in-

creased TGF- β and IL-8. Such results are intriguing and some questions arise. Does this suggest a more advanced disease or a poorer prognosis in non-responding patients? Could it represent a predictive factor of non-response for CIN patients? However, the results do not show statistically significant differences. We believe that an increased number of patients in the study could give more representative and significant differences in the cytokine and prove these findings. Research with humans and especially with human oncology is difficult because they are a susceptible study group. Another point regarding the statistical variation is that the immune system is controlled by several genetic factors, such as the histocompatibility leukocyte antigen, production of mediators, receptors and the concentrations of each one. The immune response of each patient is unique, as are some characteristics of each tumor, such as escape mechanisms and tumor antigens.

Studies based on the analyses of cervical stroma¹³ and vaginal secretion⁴² showed that IFN- α 2b treatment tended to modulate the immune response toward the Th1 pattern specifically in patients who showed a good response. In contrast, patients who had treatment failure generally showed a response toward the Treg profile.

The study of Terawaki *et al.*⁴³ proposed that strong innate inflammatory responses promote primary T-cell activation and their differentiation into effectors cells but also cause an attenuated T-cell response in sustained immune reactions, at least partially through type I IFN-mediated programmed cell death-1 (PD-1) transcription. Based on this idea, it was demonstrated that IFN- α administration in combination with PD-1 blockade in tumor-bearing mice effectively augments antitumor immunity.

Santini *et al.*⁴⁴ showed that IFN- α exerts multiple effects leading to immune protection against pathogens and cancer, as well to autoimmune reactions by acting on monocytes and dendritic cells. The versatility of human monocytes conditioned by IFN- α towards dendritic cell differentiation (IFN-DC) in shaping the autologous T-helper response was analyzed. Expansion occurring of CXCR3⁺ IFN- γ producing CD4 Th1 cells resulted in the emergence of two distinct subsets of IL-17-producing CD4 T cells: a predominant Th17 population selectively producing IL-17 and expressing CCR6; a minor Th1/Th17 population producing both IL-17 and IFN- γ . After phagocytosis of apoptotic cells, IFN-DC induced Th17 cell expansion and IL-17 release. Notably, the use of neutralizing antibodies revealed that IL-23 was an essential cytokine in mediating Th17 cell development by IFN-DC. Demonstration of the IFN-DC-induced expansion of both Th1 and Th17 cell populations reveals the intrinsic plasticity of these DC in orienting the immune response and provides a mechanistic link between IFN- α and the onset of autoimmune phenomena, which have been correlated with both IL-17 production and exposure to IFN- α .

Table 2 - Concentrations of Th1, Th2 and Treg pattern cytokines (CK) in GR and BR groups

CK ^a	GR group (n = 5)				BR group (n = 5)			
	1 st	6 th	12 th	18 th	1 st	6 th	12 th	18 th
IFN- γ	4.89 \pm 4.12	4.38 \pm 4.23	2.06 \pm 1.35	0.38 \pm 0.72	6.34 \pm 8.98	5.77 \pm 8.47	7.81 \pm 11.08	4.93 \pm 10.60
IL-1	45.75 \pm 64.36	23.09 \pm 18.80	14.92 \pm 12.56	83.19 \pm 16.6	12.56 \pm 12.71	24.88 \pm 32.18	9.72 \pm 10.81	6.27 \pm 12.73
IL-2	11.09 \pm 24.79	7.16 \pm 16.01	12.80 \pm 28.63	21.54 \pm 48.17	19.03 \pm 30.02	ND	ND	ND
IL-6	9.58 \pm 11.12	3.10 \pm 2.95	1.31 \pm 1.42	2.97 \pm 5.44	5.28 \pm 11.41	0.73 \pm 1.64	1.012 \pm 2.26	18.87 \pm 36.82
IL-8	8.65 \pm 7.53	8.11 \pm 12.45	2.52 \pm 3.15	6.12 \pm 4.47	20.70 \pm 28.48	9.31 \pm 8.58	8.56 \pm 9.97	7.42 \pm 9.31
IL-10	8.15 \pm 4.91	2.34 \pm 2.53	6.87 \pm 8.89	7.36 \pm 7.58	8.90 \pm 5.22	7.52 \pm 8.12	4.12 \pm 5.54	6.07 \pm 9.59
IL-12	333.0 \pm 417.9	805.7 \pm 479.9	1804.0 \pm 1020*	1738.0 \pm 2426.0**	425.2 \pm 239.8	602.8 \pm 139.7	391.2 \pm 722.3	448.5 \pm 407.2
TGF- β	1502.0 \pm 2169.0	2315.0 \pm 1716.0	2277.0 \pm 1510.0	2057.0 \pm 1635	3927.0 \pm 5097.0	3490.0 \pm 5605.0	2713.0 \pm 4803.0	2729.0 \pm 4020.0
TNF- α	3.66 \pm 8.18	ND	ND	0.91 \pm 2.04	6.07 \pm 13.58	4.37 \pm 9.77	7.85 \pm 17.57	4.40 \pm 9.84

^aCytokine concentration values shown in pg/ml, mean \pm SD. ND, not detected.

* $P = 0.0354$, 12th positive \times 12th negative response.

** $P = 0.0317$, 18th positive \times 18th negative response.

Recently, combination therapy of subcutaneous IFN- α and intra-arterial 5-fluorouracil showed an outstanding response rate for intractable hepatocellular carcinoma (with portal vein thrombosis). In addition, recent preclinical and clinical studies have revealed that the mechanism of combination therapy may concern direct antitumor effects (through cell-cycle arrest and induction of apoptosis) and indirect actions (through immunocompetent cells and antiangiogenic effect). For the further advance of hepatocellular carcinoma treatment and prognosis, this therapy might be a promising treatment modality and is expected to develop⁴⁵.

In conclusion, our study showed that the treatment of CIN II-III with intralesional IFN- α 2b achieved a good response in non-smoking patients and was associated with an increase in IL-12 serum levels during the period of administration.

References

- Kumar V, Abbas AK, Fausto N, Mitchell RN: Robbins Basic Pathology (8th edn), pp 718-721, Saunders Elsevier, 2007.
- World Health Organization: Human papillomavirus infection and cervical cancer, 2009. Available at http://www.who.int/vaccine_research/diseases/hpv/en/
- Arbyn M, Autier P, Ferlay J: Burden of cervical cancer in the 27 member states of the European Union: estimates for 2004. *Ann Oncol*, 18: 1423-1425, 2007.
- Arbyn M, Anttila A, Jordan J, Ronco G, Schenck U, Segnan N, Wiener H, Herbert A, von Karsa L: European Guidelines for Quality Assurance in Cervical Cancer Screening. Second Edition - Summary Document. *Ann Oncol*, 21: 448-458, 2010.
- Wright TC Jr, Massad LS, Dunton CJ, Spitzer M, Wilkinson EJ, Solomon D: American Society for Colposcopy and Cervical Pathology-Sponsored Consensus Conference. 2006 Consensus guidelines for the management of women with cervical intraepithelial neoplasia or adenocarcinoma in situ. *Am J Obstet Gynecol*, 197: 340-345, 2007.
- Jakobsson M, Gissler M, Sainio S, Paavonen J, Tapper AM: Preterm delivery after surgical treatment for cervical intraepithelial neoplasia. *Obstet Gynecol*, 109: 309-313, 2007.
- Kyrgiou M, Koliopoulos G, Martin-Hirsch P, Arbyn M, Prendiville W, Paraskeva E: Obstetric outcomes after conservative treatment for intraepithelial or early invasive cervical lesions: systematic review and meta-analysis. *Lancet*, 367: 489-498, 2006.
- Ferrantini M, Capone I, Belardelli F: Interferon-alpha and cancer: mechanisms of action and new perspectives of clinical use. *Biochimie*, 89(6-7): 884-894, 2007.
- Haller O, Kochs G, Weber F: The interferon response circuit: induction and suppression by pathogenic viruses. *Virology*, 344(1):119-130, 2006.
- Choo YC, Seto WH, Hsu C, Tany YH, Ma HK, Ng MH: Cervical intraepithelial neoplasia treated by perilesional injection of interferon. *Br J Obstet Gynaecol*, 93: 372-379, 1986.
- Dunhan AM, McCartney JC, McCance DJ, Taylor RW: Effect of perilesional injection of α -interferon on cervical intraepithelial neoplasia and associated human papillomavirus infection. *J R Soc Med*, 83: 490-492, 1990.
- Stellato G: Intralesional recombinant alpha 2B interferon in the treatment of human papillomavirus-associated cervical intraepithelial neoplasia. *Sex Transm Dis*, 19: 124-126, 1992.
- Ramos MC, Mardegan MC, Peghini BC, Adad SJ, Michelin MA, Murta EFC: Expression of cytokines in cervical stroma in patients with high-grade cervical intraepithelial neoplasia after treatment with intralesional interferon α -2b. *Eur J Gynaecol Oncol* 31(5): 522-529, 2010.
- Bell MC, Edwards RP, Partridge EE, Kuykendall K, Conner W, Gore H, Turbat-Herrera E, Crowley-Nowick PAC: CD8+ T lymphocytes are recruited to neoplastic cervix. *J Clin Immunol*, 15(3): 130-136, 1995.
- Parkin DM, Bray F, Ferlay J, Pisani P: Global cancer statistics, 2002. *CA Cancer J Clin*, 55: 74-108, 2005.
- Schoell WM, Janicek MF, Mirhashemi R: Epidemiology and biology of cervical cancer. *Semin Surg Oncol*, 16(3): 203-211, 1999.
- Castellsaqué X, Muñoz N: Chapter 3: cofactors in human papillomavirus carcinogenesis - role of parity, oral contraceptives, and tobacco smoking. *J Natl Cancer Inst Monogr*, 31: 20-28, 2003.
- Ault KA: Epidemiology and natural history of human papillomavirus infections in the female genital tract. *Infect Dis Obstet Gynecol*, suppl. 40470: 1-5, 2006.
- Mougin C, Dalstein V, Pretet JL, Gay C, Schaal JB, Riethmuller D: Epidemiology of cervical papillomavirus infections. Recent knowledge. *Presse Med*, 30(20): 1017-1023, 2001.

20. Shipitsyna E, Zolotoverkhaya E, Kuevda D, Nasonova V, Romanyuk T, Khachatryan A, Orlova O, Abashova E, Kostyuchek I, Shipulina O, Anttila A, Savicheva A: Prevalence of high-risk human papillomavirus types and cervical squamous intraepithelial lesions in women over 30 years of age in St. Petersburg, Russia. *Cancer Epidemiology*, 2010. doi:10.1016/j.canep.2010.08.010
21. Runowicz CD, Lymberis S, Tobias D: Cervical neoplasia and cigarette smoking: are they linked? *Medscape Womens Health*, 2(3): 2, 1997.
22. Tollerud DJ, Clark JW, Brown LM, Neuland CY, Mann DL, Pankiw-Trost LK, Blattner WA, Hoover RN: Association of cigarette smoking with decreased numbers of circulating natural killer cells. *Am Rev Respir Dis*, 139(1): 194, 1989.
23. Mehta H, Nazzal K, Sadikot RT: Cigarette smoking and innate immunity. *Inflamm Res*, 57: 497, 2008.
24. Stellato G: Intralesional recombinant alpha 2B interferon in the treatment of human papillomavirus-associated cervical intraepithelial neoplasia. *Sex Transm Dis*, 19: 124-126, 1992.
25. Murta EFC, Tavares Murta BM: Successful pregnancy after vaginal cancer treated with interferon. *Tumori*, 90(2): 247-248, 2004.
26. Micheletti L, Barbero M, Preti M, Zanotto Valentino MC, Nicolaci P, Corbella L, Borgno G: Intra-lesion administration of beta-interferon in the treatment of CIN associated with HPV infection. *Minerva Ginecol* 44(6): 329-334, 1992.
27. Penna C, Fallani MG, Gordigiani R, Sonni L, Taddei GL, Marchionni M: Intralesional beta-interferon treatment of cervical intraepithelial neoplasia associated with human papillomavirus infection. *Tumori*, 80(2): 146-150, 1994.
28. Cinel A, Wittenberg L, Minucci D: Beta-interferon topical treatment in low and high risk cervical lesions. *Clin Exp Obstet Gynecol*, 18(2): 91-97, 1991.
29. Sikorski M, Zrubek H: Long-term follow-up of patients treated with recombinant human interferon gamma for cervical intraepithelial neoplasia. *Int J Gynecol Obstet*, 82: 179-185, 2003.
30. Sikorski M, Bobek M, Zrubek H, Marcinkiewicz J: Dynamics of selected MHC class I and II molecule expression in the course of HPV positive CIN treatment with the use of human recombinant IFN- γ . *Acta Obstet Gynecol Scand*, 83(2): 299-307, 2004.
31. Rensing ME, Van Driel WJ, Celis E, Sette A, Brandt MP, Hartman M, Anholts JD, Schreuder GM, Ter Harmsel WB, Fleuren GJ, Trimbos BJ, Kast WM, Melief CJ: Occasional memory cytotoxic T-cell responses of patients with human papillomavirus type 16-positive cervical lesions against a human leukocyte antigen-A*0201-restricted E7-encoded epitope. *Cancer Res*, 56(3): 582-588, 1996.
32. Tsukui T, Hildesheim A, Schiffman MH, Lucci J, Contois D, Lawler P, Rush BB, Lorincz AT, Corrigan A, Burk RD, Qu W, Marshall MA, Mann D, Carrington M, Clerici M, Shearer GM, Carbone DP, Scott DR, Houghten RA, Berzofsky JA: Interleukin 2 production in vitro by peripheral lymphocytes in response to human papillomavirus-derived peptides: correlation with cervical pathology. *Cancer Res*, 56(17): 3967-3974, 1996.
33. Gaiotti D, Chung J, Iglesias M, Nees M, Baker PD, Evans CH, Woodworth CD: Tumor necrosis factor-alpha promotes human papillomavirus (HPV) E6/E7 RNA expression and cyclin-dependent kinase activity in HPV-immortalized keratinocytes by a ras-dependent pathway. *Mol Carcinog*, 27(2): 97-109, 2000.
34. De Gruijl TD, Bontkes HJ, Van Den Muysenberg AJ, Van Oostveen JW, Stukart MJ, Verheijen RH, van der Vange N, Snijders PJ, Meijer CJ, Walboomers JM, Scheper RJ: Differences in cytokine mRNA profiles between premalignant and malignant lesions of the uterine cervix. *Eur J Cancer*, 35(3): 490-497, 1999.
35. Peters JH, Gieseler R, Thiele B, Steinbach F: Dendritic cells: from oncogenetic orphans to myelomonocytic descendants. *Immunol Today*, 17(6): 273-278, 1996.
36. Clerici M, Merola M, Ferrario E, Trabattoni D, Villa ML, Stefanon B, Venzon DJ, Shearer GM, De Palo G, Clerici E: Cytokine production patterns in cervical intraepithelial neoplasia: association with human papillomavirus infection. *J Natl Cancer Inst*, 89(3): 245-250, 1997.
37. Bais AG, Beckmann I, Lindemans J, Ewing PC, Meijer CJ, Snijders PJ, Helmerhorst TJ: A shift to a peripheral Th2-type cytokine pattern during the carcinogenesis of cervical cancer becomes manifest in CIN III lesions. *J Clin Pathol*, 58(10): 1096-1100, 2005.
38. El-Sherif AM, Seth R, Tighe PJ, Jenkins D: Quantitative analysis of IL-10 and IFN-gamma mRNA levels in normal cervix and human papillomavirus type 16 associated cervical precancer. *J Pathol*, 195(2): 179-185, 2001.
39. Tavares-Murta BM, De Resende AD, Cunha FQ, Murta EFC: Local profile of cytokines and nitric oxide in patients with bacterial vaginosis and cervical intraepithelial neoplasia. *Eur J Obstet Gynecol Reprod Biol*, 138(1): 93-99, 2008.
40. Wei LH, Kuo ML, Chen CA, Cheng WF, Cheng SP, Hsieh FJ, Hsieh CY: Interleukin-6 in cervical cancer: the relationship with vascular endothelial growth factor. *Gynecol Oncol*, 82: 49-56, 2001.
41. Fujimoto J, Sakaguchi H, Aoki I, Tamaya T: Clinical implications of expression of interleukin 8 related to angiogenesis in uterine cervical cancers. *Cancer Res*, 60: 2632-2635, 2000.
42. Mardegan MC, Ramos MC, Adad SJ, Michelin MA, Shimba D, Murta EFC: Immunological evaluation of vaginal secretion in patients with high-grade cervical intra-epithelial neoplasia treated with intralesional interferon α -2b. *Eur J Gynaecol Oncol*, 32: 297-302, 2011.
43. Terawaki S, Chikuma S, Shibayama S, Hayashi T, Yoshida T, Okazaki T, Honjo T: IFN-alpha directly promotes programmed cell death-1 transcription and limits the duration of T cell-mediated immunity. *J Immunol*, 186(5): 2772-2779, 2011.
44. Santini SM, Lapenta C, Donati S, Spadaro F, Belardelli F, Ferrantini M: Interferon-a-conditioned human monocytes combine a Th1-orienting attitude with the induction of autologous Th17 Responses: role of IL-23 and IL-12. *PLoS ONE* 6(2): e17364, 2011. doi:10.1371/journal.pone.0017364
45. Nagano H: Treatment of advanced hepatocellular carcinoma: intraarterial infusion chemotherapy combined with interferon. *Oncology*, 78 (Suppl. 1): 142-147, 2010.