IMMUNOMODULATORY EFFECTS OF BCG IN PATIENTS WITH RECURRENT RESPIRATORY PAPILLOMATOSIS

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ABSTRACT

BACKGROUND: Recurrent respiratory papillomatosis (RRP) is a rare manifestation of human papilloma virus (HPV) infection with extremely high relapse frequency, poorly understood immunopathogenesis, and lack of efficient treatment. Immunotherapy with Calgevax (BCG) in combination with CO2 surgery significantly improves the outcome of RRP. The present study investigates cellular immunity parameters in RRP patients, and the effects of 20-month Calgevax immunomodulation.

MATERIALS AND METHODS: RRP patients (n = 15) subjected to combined therapy were tested before, 6, 12 and 20 months after the start of immunomodulation. Absolute counts and percentage of T, B and NK cells, effector Tc1 (CD8+IFNγ+); Th1 (CD4+IFNγ+), Th17 (CD4+IL-17+) and regulatory (CD4+FoxP3+) T lymphocytes, as well as the in vitro stimulated secretion of IL-2, IL-4, IL-5, IL-10, IFNγ and TNFα were determined by flow cytometry (FACSCanto II, BD).

RESULTS: While no significant changes were detected in the circulating T, B and NK subsets, RRP patients presented increased proportions of Tc1, Th1 and Th17 cells, and significantly reduced IFNγ/IL-4 and IFNγ/IL-10 ratios as compared to healthy controls (15% vs. 8%), (58 vs. 139 and 15 vs. 26, respectively), p < 0.05 for all comparisons. Increased Treg (9% vs. 4%), and decreased Th17 effectors share (0.7% vs. 0.4%) were observed at 12 months, while IFNγ/IL-4 and IFNγ/IL-10 ratios were restored after 20 months of Calgevax application.

CONCLUSIONS: Antiviral response closely depends on cytokine background. Calgevax potentiates Treg differentiation at the expense of proinflammatory Th17, limits hyperactivation and virus-specific T cell clones depletion, and restores a Th1 cytokine background.

Key words: RRP, HPV, Calgevax, T-cell response

INTRODUCTION

Recurrent respiratory papillomatosis (RRP) caused by human papillomavirus (HPV) is characterized by proliferation of premalignant squamous papillomas within the respiratory tract. Although over 90% of the cases are associated with low-risk strains (type 6 and 11), the disease has often dramatic presentation due to significant airway obstruction, debilitating frequency of relapses after standard surgery, and unpredictable prognosis.1,2 Malignant degeneration to squamous cell carcinoma with very poor prognosis is observed in 3-5% of patients. Extension into the lower airways occurs in approximately 17% of patients.3,4 Having in mind that asymptomatic HPV infection is very common among the population, the low incidence of RRP raises the question about the immune mechanisms that might determine the occurrence, relapses, and malignisation.5 Contemporary CO2 laser microsurgery is sparing but does not prevent relapses. Multiple combined protocols including antivirals (ribavirin and cidofovir), vitamins and oligoelements, cyclooxygenase-2, EGFR or IFNγ have been applied without definitive success.1

Efficient control of viral infections comprises imminent NK cell activation; followed by the differentiation of IFNγ producing CD4 (Th1) and CD8 T cell effectors. In addition, regulatory CD4+FoxP3+ T cells with inhibitory function (Treg) prevent the terminal effector cell differentiation in case of extreme immune activation, and potentiate immune memory.5 However, persistent infections are...
often associated with a misbalanced differentiation of proinflammatory, effector and Treg cells, and disrupted Th1/Th2/Th17 cytokine balance. The Bulgarian preparation Calgevax (BB-NCIPD Ltd) contains freeze-dried live bacteria derived from BCG culture. It is a potent stimulator of Th1 responses, and has been successfully applied for treatment of superficial bladder tumors, and malignant melanoma.

Data about the effects of BCG in HPV infection are still scarce. We have applied for the first time combined CO₂ microsurgery / Calgevax therapy in RRP patients that has resulted in a significant reduction of relapses.

AIM

The present study aims at delineating the characteristics of the inefficient cellular immune response in RRP, and investigates the effects of Calgevax (BCG) on the T cell response of patients subjected to combined surgery / immune modulation.

MATERIALS AND METHODS

RRP patients (n = 15, 9 men, 6 women, mean age 35.2 years) were subjected to CO₂ laser microsurgery followed by immunotherapy. Calgevax (2.56 x 10⁸ CFU) was administered by scarification (on a 25-cm² skin area) in 12 applications at 40-45-day intervals comprising a period of 18 to 20 months. The control group included age- and sex-matched healthy individuals fully conforming to the CLSI standards. Informed consent approved by the local ethical committee was obtained from all participants. Immune parameters were defined before (0), 6, 12 and 20 months after the start of immune modulation. Percentages of peripheral blood lymphocyte subsets were studied by multicolor flow cytometry (FACSCanto II, BD), absolute counts were determined after a standard lysis-no-wash procedure using TRUCount tubes (BD Biosciences). Treg (CD4+CD25hi+FoxP3+) were determined by FoxP3 intracellular staining (BD FoxP3 Staining Kit). Percentages of effector Tc1 (CD8+CD69+IFNγ+), Th1 (CD4+CD69+IFNγ+) and Th17 (CD4+CD69+IL-17+) cells were determined after overnight in vitro PHA stimulation and intracellular IFNγ staining. The in vitro PHA-stimulated secretion of IL-2, IL-4, IL-5, IL-10, IFNγ and TNFα was measured by flow cytometry (BD CBA kit). Intragroup and intergroup comparisons were performed with appropriate nonparametric statistical methods and software GraphPad, v. 5.

RESULTS

The baseline proportions and absolute counts of T (CD3+CD56-), B (CD19+CD3-) and NK (CD56+CD16+CD3-) cells in the patients group corresponded to the reference ranges for the Bulgarian population and did not change significantly in the course of Calgevax treatment (data not shown). Further on, no significant differences were detected for the CD4 (Fig. 1A) and CD8 T subsets (Fig. 1B) as compared to healthy controls. Therefore, we looked for more subtle changes at the T cell subset level.

Significantly increased levels of IFNγ-secreting CD4 (Th1) and CD8 (Tc1) cells were established in untreated RRP patients in response to non-specific stimulation with PHA as compared to healthy controls (mean 9.3% vs. 5.7% and 15.7% vs. 8.4 %, respectively, p < 0.05 for both). After 20 months of Calgevax treatment Th1 levels were within control reference interval while high Tc1 levels persisted (Figs 2A, B). Importantly, at 6 months of immunotherapy the share of Th17 cells significantly decreased (0.4% vs. 0.7%), (Fig. 2C) and at 12 months the level of circulating Treg cells significantly increased as compared to baseline and healthy controls (8% vs. 4.5% and 3.8%, respectively). This increase was followed by normalization to control group levels at the end of the study period (Fig. 2D).

To evaluate the functional consequences of the changes observed at the subset level, we studied the basic Th1 (IFNγ, TNFa, IL-2), Th2 (IL-4, IL-5) and regulatory (IL-10) cytokines secreted in response to non-specific stimulation. We found that IFNγ/IL-4 and IFNγ/IL-10 ratios characterizing the effective antiviral response, were significantly reduced at baseline (58 vs. 139 and 15 vs. 26, respectively, p < 0.05 for both), but were restored to control levels (159 vs. 139 and 28 vs. 26, respectively, p > 0.05 for both) after 20 months of Calgevax application (Figs 3A, B).
Figure 1. Proportions of CD4 and CD8 T cell subsets in RRP patients. Percentages of Th (CD4+CD3+) and Tc (CD8+CD3+) cells were determined in blood samples from RRP patients before (0), 6, 12 and 20 months after the start of Calgevax application in comparison to healthy controls. The thick and dotted lines correspond to the mean (min/max) reference values.

Figure 2. Effector and regulatory T cell subsets in RRP patients. Percentages of A: Th1 (CD4+ CD69+IFNγ+), B: Tc1 (CD8+ CD69+IFNγ+), C: Th17 (CD4+IL-17+) cells after 12h in vitro PHA stimulation of whole blood samples and D: CD4+CD25highFoxP3+ T lymphocytes (Treg) from RRP patients before (0), 6, 12 and 20 months after the start of Calgevax treatment. The thick and dotted lines correspond to the mean (min/max) control values. * Significant difference in comparison with month 20. Wilcoxon p < 0.05, ** Significant difference in comparison with healthy controls, Man-Witney p < 0.05.
toxicity, phagocytosis, and antigen-specific response mediated by CD4 Th1 and cytotoxic CD8 (Tc1) T lymphocytes.\textsuperscript{9,11} In line with published data,\textsuperscript{11} the background levels of the basic lymphocyte subsets in RRP patients revealed no apparent immune deficiency. Further on, no significant changes were detected for CD4 and CD8 T cells and their ratio, indicating more subtle defects.

Accumulating evidence from animal models and in vitro studies suggests that Th cell subsets are not irreversibly differentiated, but can exhibit plasticity by changing transcription factor expression or by expressing multiple transcription factors. This plasticity has been recently suggested to play a role in modulating immune response during inflammation.\textsuperscript{12}

Our results show that RRP is associated with disturbed cytokine balance, characterized with predominance of Th17 and Th2 at the expense of Th1-type cytokines. The role of Th1 cytokines IFNg and IL-12 for the activation of natural cytotoxic mechanisms, the maturation of antigen-presenting dendritic cells and the efficient activation and differentiation of adaptive anti-viral effectors is well established.\textsuperscript{11,13} Further on, the Th1 cytokine IL-2 is vital for the differentiation of Treg with inhibitory function that maintain an effector / memory T cell balance in conditions of persistent immune stimulation as a hallmark of protective immune response.\textsuperscript{5} On the other hand, a Th2-biased adaptive immune response has been reported in RRP. Both IL-10 and IL-4 are upregulated in papillomas and in peripheral blood mononuclear cells exposed to HPV-11 E6 protein, with a concomitant decrease of IFN-\gamma, IL-12 and IL-18 expression. This impaired cytokine balance correlates with disease severity.\textsuperscript{14,15} Besides, Th2-like chemokines CCL17, CCL18 and CCL22 were found to be elevated in RRP and reduced in concert with sustained clinical remission.\textsuperscript{15}

The role of Th17 in chronic viral infections remains controversial. Both protective and pro-inflammatory effects have been assigned to Th17 effectors, but there is growing evidence that Th17 cells are pathological in many human autoimmune and inflammatory diseases, leading to intense interest in defining their origins, functions and developing strategies to block their pathological effects.\textsuperscript{16-18} It has been demonstrated that the skewing of murine Th towards Th17 and Treg is mutually exclusive, the Th17/Treg balance being critical for immune homeostasis.\textsuperscript{18,19} Treg have been associated with both reduced antiviral T-cell responses, and with control of generalized T cell activation.\textsuperscript{5}

Our results suggest that BCG may beneficially affect the differentiation and balance of the effector and regulatory T cell subsets in RRP patients by changing the cytokine background. In bladder cancer immunotherapy, BCG stimulation has been shown to elevate IL-2, TNF and INF-\gamma levels and activate Th1 cells necessary for cytotoxic T lymphocyte differentiation.\textsuperscript{20} In our hands, Calgevax
potentiated the differentiation of Treg at the expense of proinflammatory Th17 cells. Importantly, Treg increase was temporary and did not limit cytotoxic CD8 response, as witnessed by high Tc1 levels and increased IFNγ/IL4 and IFNγ/IL10 ratios in the end of the study period. We may speculate that the actual effect of Treg is to limit the immune hyperactivation, prevent the depletion of virus-specific CD8 and CD4 T cell clones and potentiate the formation of virus-specific memory.

CONCLUSIONS

RRP is characterized with prevalence of Th17 and Th2 at the expense of Th1-type cytokines. BCG increases the efficiency of antiviral T-cell response by restoring Th1/Th2/Th17 cytokine balance and inducing the differentiation of Treg. The latter prevent the exhaustion of effector clones in the settings of chronic infection and promote the development of protective T cell immune response.

REFERENCES

ИММУНОМОДЕЛИРУЮЩИЕ ЭФФЕКТЫ BCG У ПАЦИЕНТОВ С РЕЦИДИВИРУЮЩИМ РЕСПИРАТОРНЫМ ПАПИЛЛОМАТОЗОМ

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РЕЗЮМЕ

ЦЕЛЬ: Рецидивирующий респираторный папилломатоз (РРП), вызванный человеческим папилломавирусом (HPV), характеризуется тяжелой клиникой и частыми рецидивами, слабо изученным патогенезом и отсутствием эффективного лечения. Иммуномодуляция посредством болгарского препарата „Calgevax“ (BCG) в комбинации с CO₂ лазерной микрохирургией значительно улучшает исход РРП. Настоящее исследование изучает эффект 20-месячной иммуномодуляции посредством „Calgevax“ (BCG) на параметры клеточного иммунного ответа у пациентов с РРП.

МАТЕРИАЛ И МЕТОДЫ: Пациенты с РРП (n=15), подвергнутые комбинированной хирургической и иммуномодулирующей терапией с помощью „Calgevax“ по схеме, обследованы до, на 6-ой, 12-й и 20-й мес. после начала иммунотерапии. Абсолютное число и процент T, B и NK клеток, процент эффекторных Tc1 (CD8+IFNγ+); Th1 (CD4+IFNγ+), Th17 (CD4+IL-17+) и регуляторных (CD4+FoxP3+) T лимфоцитов (Treg), как и in vitro стимулированная секреция IL-2, IL-4, IL-5, IL-10, IFNγ и TNFα определены флуометрически (FACSCanto II, BD).

РЕЗУЛЬТАТЫ: Процент циркулирующих T, В, NK клеток у РРП пациентов не отличается значимо от стоимости у здоровых людей. Нелеченные пациенты с РРП характеризуются повышенными уровнями Tc1 (15% vs. 8%), Th1(9% vs. 6%), и Th17(0.7% vs. 0.56%) клеток и значимо уменьшенными стоимостями соотношений IFNγ/IL-4 (58 vs. 139) и IFNγ/IL-10 по сравнению со здоровыми лицами контрольной группы (38 vs. 139 и 15 vs. 26 соответственно), p<0.05 при всех сравнениях. Увеличенный уровень Treg (9% vs. 4%) и уменьшенная часть Th17 эффекторов (0.7% vs.0.4%) установлены на 12-й мес., а соотношения IFNγ/IL-4 и IFNγ/IL-10 восстанавливаются на 20-й мес. от терапии препаратом „Calgevax“.

ВЫВОДЫ: Эффективность антивирусного ответа зависит от фона цитокинов. „Calgevax“ стимулирует секрецию Th1 цитокинов (IFNγ, IL-2), чем потенцирует дифференциацию Treg за счет проинфламматорных Th17 и предотвращает углубление иммунного воспаления, предотвращает сверхстимуляцию и истощение вирус-специфических T клеточных субпопуляций.