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NLGP counterbalances the immunosuppressive effect of tumor-associated mesenchymal stem cells to restore effector T cell functions

Tithi Ghosh, Partha Nandi, Nilanjan Ganguly, Ipsita Guha, Avishek Bhuniya, Sarbari Ghosh, Anirban Sarkar, Akata Saha, Shayani Dasgupta, Rathindranath Baral and Anamika Bose*

Abstract

Background: A dynamic interaction between tumor cells and its surrounding stroma promotes the initiation, progression, metastasis, and chemoresistance of solid tumors. Emerging evidences suggest that targeting the stromal events could improve the efficacies of current therapeutics. Within tumor microenvironment (TME), stromal progenitor cells, i.e., MSCs, interact and eventually modulate the biology and functions of cancer and immune cells. Our recent finding disclosed a novel mechanism stating that tumor-associated MSCs inhibit the T cell proliferation and effector functions by blocking cysteine transport to T cells by dendritic cells (DCs), which makes MSCs as a compelling candidate as a therapeutic target. Immunomodulation by nontoxic neem leaf glycoprotein (NLGP) on dysfunctional cancer immunity offers significant therapeutic benefits to murine tumor host; however, its modulation on MSCs and its impact on T cell functions need to be elucidated.

Methods: Bone marrow-derived primary MSCs or murine 10 T1/2 MSCs were tumor-conditioned (TC-MSCs) and co-cultured with B16 melanoma antigen-specific DCs and MACS purified $CD4^+$ and $CD8^+$ T cells. T cell proliferation of T cells was checked by Ki67-based flow-cytometric and thymidine-incorporation assays. Cytokine secretion was measured by ELISA. The expression of cystathionase in DCs was assessed by RT-PCR. The STAT3/pSTAT3 levels in DCs were assessed by western blot, and STAT3 function was confirmed using specific SiRNA. Solid B16 melanoma tumor growth was monitored following adoptive transfer of conditioned $CD8^+$ T cells.

Results: NLGP possesses an ability to restore anti-tumor T cell functions by modulating TC-MSCs. Supplementation of NLGP in DC-T cell co-culture significantly restored the inhibition in T cell proliferation and IFNγ secretion almost towards normal in the presence of TC-MSCs. Adoptive transfer of NLGP-treated TC-MSC supernatant educated $CD8^+$ T cells in solid B16 melanoma bearing mice resulted in better tumor growth restriction than TC-MSC conditioned $CD8^+$ T cells. NLGP downregulates IL-10 secretion by TC-MSCs, and concomitantly, pSTAT3 expression was downregulated in DCs in the presence of NLGP-treated TC-MSC supernatant. As pSTAT3 negatively regulates cystathionase expression in DCs, NLGP indirectly helps to maintain an almost normal level of cystathionase gene expression in DCs making them able to export sufficient amount of cysteine required for optimum T cell proliferation and effector functions within TME.

(Continued on next page)

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ORIGINAL RESEARCH
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Neem Leaf Glycoprotein Restrains VEGF Production by Direct Modulation of HIF1α-Linked Upstream and Downstream Cascades

*Akata Saha, Partha Nandi, Shayani Dasgupta, Avishek Bhuniya, Nilanjan Ganguly, Tithi Ghosh, Ipsita Guha, Saptak Banerjee, Rathindranath Baral and Anamika Bose**

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Neem Leaf Glycoprotein (NLGP) is a natural immunomodulator, have shown sustained tumor growth restriction as well as angiogenic normalization chiefly by activating $CD8^+$ T cells. Here, we have investigated the direct role of NLGP as a regulator of tumor microenvironmental hypoxia and associated vascular endothelial growth factor (VEGF) production. We observed a significant reduction in VEGF level in both *in vivo* murine tumor and *in vitro* cancer cells (B16Mel, LLC) and macrophages after NLGP treatment. Interestingly, NLGP mediated VEGF downregulation in tumor cells or macrophages within hypoxic chamber was found at an early 4 h and again at late 24 h in mRNA level. Our data suggested that NLGP prevented hypoxia-induced strong binding of HIF1α with its co-factors, CBP/p300 and Sp3, but not with Sp1, which eventually limit the binding of HIF1α-transcriptional complex to hypoxia responsive element of VEGF promoter and results in restricted early VEGF transcription. On the otherhand, suppressed phosphorylation of Stat3 by NLGP results reduction of HIF1α at 24 h of hypoxia that further support sustained VEGF down-regulation. However, NLGP fails to regulate VHL activity as observed by both *in vivo* and *in vitro* studies. Therefore, this study for the first time reveals a mechanistic insight of NLGP mediated inhibition of angiogenesis by suppressing VEGF, which might help in vascular normalization to influence better drug delivery.

Keywords: hypoxia, VEGF, HIF1α, STAT3, tumor-microenvironment, NLGP

SUMMARY

NLGP downregulates VEGF at 4 h transcriptionally and both VEGF and HIF1α at 18 h translationally. Co-factors of HIF1α-transcriptional-complex downregulated at 4 h in hypoxia by NLGP. Downregulation of pStat3 by NLGP downregulates HIF1α resulting in a sustained VEGF reduction in hypoxia.

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NLGP Attenuates Murine Melanoma and Carcinoma Metastasis by Modulating Cytotoxic CD8$^+$ T Cells

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Neem leaf glycoprotein (NLGP), a natural immunomodulator, attenuates murine carcinoma and melanoma metastasis, independent of primary tumor growth and alterations in basic cellular properties (cell proliferation, cytokine secretion, etc.). Colonization event of invasion–metastasis cascade was primarily inhibited by NLGP, with no effect on metastasis-related invasion, migration, and extravasation. High infiltration of interferon γ (IFN-γ)–secreting cytotoxic CD8$^+$ T cells [CD44$^+$, CD69$^+$, GranB$^+$, IFN-γ$^+$, and interleukin 2$^+$] was documented in the metastatic site of NLGP-treated mice. Systemic CD8$^+$ T cell depletion abolished NLGP-mediated metastasis inhibition and reappeared upon adoptive transfer of NLGP-activated CD8$^+$ T cells. Interferon γ-secreting from CD8$^+$ T cells inhibit the expression of angiogenesis regulatory vascular endothelial growth factor and transforming growth factor β and have an impact on the prevention of colonization. Neem leaf glycoprotein modulates dendritic cells (DCs) for proper antigen presentation by its DC surface binding and upregulation of MHC-I/II, CD86, and CCR7. Neem leaf glycoprotein–treated DCs specifically imprint CXCR3 and CCR4 homing receptors on activated CD8$^+$ T cells, which helps to infiltrate into metastatic sites to restrain colonization. Such NLGP's effect on DCs is translation dependent and transcription independent. Studies using ovalbumin, $OVA_{257-264}$, and crude B16F10 antigen indicate MHC-I upregulation depends on the quantity of proteasome degradable peptide and only stimulates CD8$^+$ T cells in the presence of antigen. Overall data suggest NLGP inhibits metastasis, in conjunction with tumor growth restriction, and thus might appear as a promising next-generation cancer immunotherapeutic.

Keywords: antigen presentation, B16F10, CD8$^+$ T cells, dendritic cells, LLC, metastasis, metastatic colonization, NLGP

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INTRODUCTION

Metastasis, an eminent hallmark of cancer (1), is an inefficacious and cumbersome expedition of tumor cells from primary to the secondary site(s) (2, 3) causing 90% cancer mortality worldwide (3). The invasion–metastasis cascade defines all the steps involved in metastasis formation; among them, successful colonization or macrometastasis formation is the last and crucial step for clinical

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ORIGINAL ARTICLE

PKCζ mediated anti-proliferative effect of C2 ceramide on neutralization of the tumor microenvironment and melanoma regression

Sweta Ghosh[1] · Subir Kumar Juin[1] · Partha Nandi[2] · Suchandra Bhattacharyya Majumdar[1] · Anamika Bose[2] · Rathindranath Baral[2] · Parames C. Sil[1] · Subrata Majumdar[1]

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Abstract

Immunotherapy, which has advantages over chemotherapy due to lesser toxicity and higher specificity, is on the rise to treat cancer. Recently, pro-apoptotic glycolipid, ceramide has emerged as a key regulator in cancer immunotherapy. The present study elucidated the potential anti-melanoma efficacy of cell-permeable, exogenous C2 ceramide on cell death and amelioration of tumor microenvironment (TME). We, for the first time, demonstrated that C2 ceramide triggered apoptosis of melanoma cells by augmenting PKCζ along with pro-inflammatory cytokines and signaling factors. C2 ceramide showed a PKCζ-mediated tumor-suppressive role in melanoma without exhibiting hepatotoxicity and nephrotoxicity. Moreover, PKCζ was revealed as one of the key regulators of Akt and ceramide during C2 ceramide-mediated apoptosis. C2 ceramide was effective in repolarization of M2 macrophage phenotype and reduction of angiogenic factors such as VEGF, VEGFR1, VEGFR2, HIF1α. Interestingly, PKCζ knockdown attenuated C2 ceramide-mediated inhibition of melanoma progression. Restoration of the Th1 type TME by C2 ceramide enhanced cytotoxic T cell-mediated killing of melanoma cells. Altogether, the study unraveled that C2 ceramide-induced PKCζ was associated with favorable immune cell functioning in TME leading to melanoma regression. Thus, our findings explored a novel mechanistic insight into C2 ceramide as a promising immunotherapeutic agent in melanoma treatment.

Keywords Immunotherapy · Melanoma · C2 ceramide · Tumor microenvironment · PKCζ · Apoptosis

Abbreviations

CM	Conditioned medium
HGF	Hepatocyte growth factor
HIF1α	Hypoxia-inducible factor 1-alpha
MSC	Mesenchymal stromal cells
PDGF	Platelet-derived growth factor
PKC	Protein kinase C
PKCζ	Protein kinase C zeta
TADC	Tumor-associated dendritic cells
TAM	Tumor-associated macrophage

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Introduction

Cancer, one of the most progressive and devastating diseases of the current world, causes an ample amount of death each year [1, 2]. The majority of metastatic cancers are resistant to diverse chemotherapeutic agents or the drugs are having adverse effects [3, 4]. Hence, the long-term survivability of patients is lamentable. Among the different types of cancers, melanoma is one of the most common forms occurring worldwide [5–7]. Melanoma is an uncontrolled cutaneous proliferation which is characterized by abnormal growth of normal melanocytes and the ability of the modified melanocytes to invade the basement membrane of the skin [8]. Recent therapies of cancer majorly involve the use of cytotoxic drugs which are not very specific and, therefore, cause harm to the normal cells and immunocytes resulting

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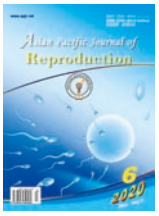

Original Article

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The leaf extracts of *Camellia sinensis* (green tea) ameliorate sodium fluoride−induced oxidative stress and testicular dysfunction in rats

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ABSTRACT

Objective: To investigate the effect of *Camellia* (*C.*) *sinensis* in mitigating oxidative damage and reproductive toxicity in testis induced by sodium fluoride in a rat model.

Methods: Twenty-four adult male Wister rats were divided into 4 groups, with 6 rats in each group. Group 1 orally received distilled water (1 mL/100 g body weight) daily and served as the control group, while group 2 received drinking water with 100 ppm sodium fluoride per day for 21 consecutive days, group 3 was administered with only *C. sinensis* extract by gavage at a dose of 100 mg/kg body weight and group 4 received drinking water with 100 ppm sodium fluoride and 100 mg/kg body weight *C. sinensis* leaf extract per day for 21 consecutive days. At the end of the treatment, the rats were sacrificed under light ether anesthesia. The gonado-somatic index, sperm count and motility, serum level of luteinizing hormone and testosterone were assayed. Lipid peroxidation [malondialdehyde (MDA) level], nitric oxide (NO) production, and activities of antioxidant enzymes - superoxide dismutase (SOD), catalase, and reduced glutathione level (GSH) were also analysed.

Results: Sodium fluoride treatment significantly decreased gonado-somatic index, sperm count and motility as well as the serum level of luteinizing hormone and testosterone (P<0.05). The histological examination of testes revealed atrophy and degenerative changes in several seminiferous tubules, along with enhanced interstitial space and a reduced number of Leydig cells. There was a highly significant increase in NO and MDA production (P<0.05), while SOD, catalase activities and GSH level decreased significantly (P<0.05). However, *C. sinensis* significantly restored testicular weight, sperm parameter, hormonal level (P<0.05), and also reversed MDA and NO generation and antioxidant enzymes activities in the testicular tissue (P<0.05).

Conclusions: *C. sinensis* may have an ameliorative role against sodium fluoride-induced oxidative damage in the testis probably because of its antioxidant property.

KEYWORDS: Oxidative stress; Testicular damage; *Camellia sinensis*; Antioxidant; Sodium fluoride

1. Introduction

Infertility is a reproductive disorder, defined by the inability to achieve a pregnancy after one year of unprotected intercourse. According to World Health Organization, the prevalence of infertility is between 3.9% and 16.8% in India. Various environmental factors threaten the reproductive health and one such is the contamination of drinking water with fluoride[1]. Fluoride, a bone-seeking element, is now regarded as a potential contributing factor to a growing male infertility in humans. And consistent with this, previous studies, both experimental and epidemiological, have uncovered that long term fluoride exposure can induce a profound negative impact on male reproductive health and reduce fertility by changing sperm quality[2], preventing spermatogenesis, testicular degeneration[3] and androgenesis[4]. Different studies on many species of animals (both *in vivo* and *in vitro*) also demonstrated that fluoride can pass through the blood-testis barrier and the blood-epididymis barriers[5] and thereby seriously affect sperm physiology and functionality such as reduction in the sperm number, movement, normal morphology and fertilizing potential of sperms[3,6,7]. In addition, fluoride can readily damage the histological structure of the testis[8,9] and epididymis which adversely affects spermatogenesis and maturation as well as fertilizing potential of sperm[10,11]. Besides, the smooth progress of spermatogenesis and growth of sexual organs essentially in males require testosterone, a steroid hormone[12] and previous studies

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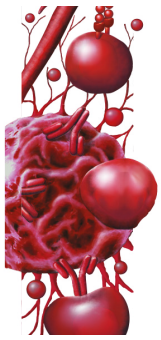
Research Article

Immunotherapy



Neem leaf glycoprotein reverses tumor-induced and age-associated thymic involution to maintain peripheral CD8$^+$ T cell pool

Ipsita Guha[1], Avishek Bhuniya[1], Partha Nandi[1], Shayani Dasgupta[1], Anirban Sarkar[1], Akata Saha[1], Juhina Das[1], Nilanjan Ganguly[1], Sarbari Ghosh[1], Tithi Ghosh[1], Madhurima Sarkar[1], Sweta Ghosh[2], Subrata Majumdar[2], Rathindranath Baral[‡,1] & Anamika Bose*[,‡,1]

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Aim: As tumor causes atrophy in the thymus to target effector-T cells, this study is aimed to decipher the efficacy of neem leaf glycoprotein (NLGP) in tumor- and age-associated thymic atrophy. **Materials & methods:** Different thymus parameters were studied using flow cytometry, reverse transcriptase PCR and immunocyto-/histochemistry in murine melanoma and sarcoma models. **Results:** Longitudinal NLGP therapy in tumor hosts show tumor-reduction along with significant normalization of thymic alterations. NLGP downregulates intrathymic IL-10, which eventually promotes Notch1 to rescue blockade in CD25$^+$CD44$^+$c-Kit$^+$DN2 to CD25$^+$CD44$^-$c-Kit$^-$DN3 transition in T cell maturation and suppress Ikaros/IRF8/Pu.1 to prevent DN2-T to DC differentiation in tumor hosts. The CD5^{int}TCR$\alpha\beta^{high}$ DP3 population was also increased to endorse CD8$^+$ T cell generation. **Conclusion:** NLGP rescues tumor-induced altered thymic events to generate more effector T cells to restrain tumor.

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Keywords: dendritic cells • IL-10 • NLGP • thymic atrophy • thymus • tumor

The cellular immune system in the tumor microenvironment not only fails to mount an effective antitumor response, but also actively enhances the intimacy with transformed cells to promote tumorigenesis [1]. On the other hand, immunotherapy enhances altered host immune response to combat against cancer [2]. The main arsenal in both scenarios is thymus differentiated T cells. Being a site for T cell differentiation, the thymus becomes targeted in cancer, though it is known to start to involute after puberty and is believed to become nonfunctional in adults. Contrary to the traditional view, recent studies with adult thymus suggest the thymic environment is maintained throughout the life [3] and inflammatory diseases such as cancer can initiate thymopoiesis, though mainly contribute Tregs expansion rather than effector T cells [4].

In context to the tumor, several *in vivo* and *in vitro* studies with tumor-induced thymic alterations observed: alterations in thymic size and cellularity; accumulation of early CD4$^-$CD8$^-$ double negative (DN)-pro T cells; enhanced apoptosis of immature CD4$^+$CD8$^+$ double positive (DP) thymocytes; loss of CD8$^+$ single positive (SP) thymocytes; alterations of thymic cytokine/chemokine gradient. All these events cumulatively diminish antitumor effector CD8$^+$ T cell pool to ultimately promote impaired cellular immunity.

Neem leaf glycoprotein (NLGP) is a neem-derived natural nontoxic immunomodulator, exhibits robust antitumor activity, chiefly by activating CD8$^+$ T cells as reported in several murine tumor models [5–7]. Corrolarily, NLGP-mediated tumor growth restriction is associated with reduction of immune-suppressor cells (regulatory T cells, tumor-associated macrophages, myeloid-derived suppressor cells and dendritic cells) [8–11] and vascular

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Tumor Arrests DN2 to DN3 Pro T Cell Transition and Promotes Its Conversion to Thymic Dendritic Cells by Reciprocally Regulating Notch1 and Ikaros Signaling

Ipsita Guha[1], Avishek Bhuniya[1], Divanshu Shukla[2†], Ashok Patidar[2†], Partha Nandi[1], Akata Saha[1], Shayani Dasgupta[1], Nilanjan Ganguly[1], Sweta Ghosh[3], Arathi Nair[2], Subrata Majumdar[3], Bhaskar Saha[2], Walter J. Storkus[4], Rathindranath Baral[1‡] and Anamika Bose[1‡]*

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Tumor progression in the host leads to severe impairment of intrathymic T-cell differentiation/maturation, leading to the paralysis of cellular anti-tumor immunity. Such suppression manifests the erosion of $CD4^+CD8^+$ double-positive (DP) immature thymocytes and a gradual increase in $CD4^-CD8^-$ double negative (DN) early T-cell progenitors. The impact of such changes on the T-cell progenitor pool in the context of cancer remains poorly investigated. Here, we show that tumor progression blocks the transition of $Lin^-Thy1.2^+CD25^+CD44^+c\text{-}Kit^{low}DN2b$ to $Lin^-Thy1.2^+CD25^+CD44^-c\text{-}Kit^-DN3$ in T-cell maturation, instead leading to DN2-T-cell differentiation into dendritic cells (DC). We observed that thymic IL-10 expression is upregulated, particularly at cortico-medullary junctions (CMJ), under conditions of progressive disease, resulting in the termination of $IL\text{-}10R^{high}$ DN2-T-cell maturation due to dysregulated expression of Notch1 and its target, CCR7 (thus restricting these cells to the CMJ). Intrathymic differentiation of T-cell precursors in $IL\text{-}10^{-/-}$ mice and *in vitro* fetal thymic organ cultures revealed that IL-10 promotes the interaction between thymic stromal cells and $Notch1^{low}$ DN2-T cells, thus facilitating these DN2-T cells to differentiate toward $CD45^+CD11c^+MHC\text{-}II^+$ thymic DCs as a consequence of activating the Ikaros/IRF8 signaling axis. We conclude that a novel function of thymically-expressed IL-10 in the tumor-bearing host diverts T-cell differentiation toward a DC pathway, thus limiting the protective adaptive immune repertoire.

Keywords: thymus, T cell, IL-10, DN2b, DC, Cancer

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Cell Death & Differentiation

ARTICLE

RGS5–TGFβ–Smad2/3 axis switches pro- to anti-apoptotic signaling in tumor-residing pericytes, assisting tumor growth

Shayani Dasgupta[1] · Tithi Ghosh[1] · Jesmita Dhar[2] · Avishek Bhuniya[1] · Partha Nandi[1] · Arnab Das[1,3] · Akata Saha[1] · Juhina Das[1] · Ipsita Guha[1] · Saptak Banerjee[1] · Mohona Chakravarti[1] · Partha Sarathi Dasgupta[1] · Neyaz Alam[4] · Jayanta Chakrabarti[4] · Subrata Majumdar[5] · Pinak Chakrabarti[2] · Walter J. Storkus[6] · Rathindranath Baral[1] · Anamika Bose[1]

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Abstract

Regulator-of-G-protein-signaling-5 (RGS5), a pro-apoptotic/anti-proliferative protein, is a signature molecule of tumor-associated pericytes, highly expressed in several cancers, and is associated with tumor growth and poor prognosis. Surprisingly, despite the negative influence of intrinsic RGS5 expression on pericyte survival, RGS5^{high}pericytes accumulate in progressively growing tumors. However, responsible factor(s) and altered-pathway(s) are yet to report. RGS5 binds with Gαi/q and promotes pericyte apoptosis in vitro, subsequently blocking GPCR-downstream PI3K-AKT signaling leading to Bcl2 downregulation and promotion of PUMA-p53-Bax-mediated mitochondrial damage. However, within tumor microenvironment (TME), TGFβ appeared to limit the cytocidal action of RGS5 in tumor-residing RGS5^{high}pericytes. We observed that in the presence of high RGS5 concentrations, TGFβ–TGFβR interactions in the tumor-associated pericytes lead to the promotion of pSmad2–RGS5 binding and nuclear trafficking of RGS5, which coordinately suppressed RGS5–Gαi/q and pSmad2/3–Smad4 pairing. The RGS5–TGFβ–pSmad2 axis thus mitigates both RGS5- and TGFβ-dependent cellular apoptosis, resulting in sustained pericyte survival/expansion within the TME by rescuing PI3K-AKT signaling and preventing mitochondrial damage and caspase activation. This study reports a novel mechanism by which TGFβ fortifies and promotes survival of tumor pericytes by switching pro- to anti-apoptotic RGS5 signaling in TME. Understanding this altered RGS5 signaling might prove beneficial in designing future cancer therapy.

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Introduction

Disorganized blood vasculature is a cardinal feature of all rapidly growing solid tumors, contributing to metastasis and resistance to therapy [1–3]. Tumor-resident perivascular cells (i.e., pericytes) exhibit loose attachment with endothelium, resulting in a leaky, chaotic vasculature that limits tissue perfusion and promotes hypoxia within the tumor microenvironment (TME) [4]. Tumor pericytes express high levels of platelet-derived-growth-factor-receptor-β (PDGFRβ), regulator-of-G-protein-signaling-5 (RGS5), neuron-glial-2 (NG2), and low levels of α-smooth-muscle-actin (SMA) representing immature phenotype [5]. Among various molecules, RGS5 has been identified as signature marker of angiogenic tumor pericytes [4, 5].

RGS5 belongs to B/R4 sub-family of RGS-proteins that modulate G-protein-coupled-receptors (GPCRs) signaling by accelerating intrinsic GTPase activity of Gα subunit of

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The immunomodulatory impact of naturally derived neem leaf glycoprotein on the initiation progression model of 4NQO induced murine oral carcinogenesis: a preclinical study

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Introduction: Murine tumor growth restriction by neem leaf glycoprotein (NLGP) was established in various transplanted models of murine sarcoma, melanoma and carcinoma. However, the role of NLGP in the sequential carcinogenic steps has not been explored. Thus, tongue carcinogenesis in Swiss mice was induced by 4-nitroquinoline-1-oxide (4NQO), which has close resemblance to human carcinogenesis process. Interventional role of NLGP in initiation-promotion protocol established during 4NQO mediated tongue carcinogenesis in relation to systemic immune alteration and epithelial-mesenchymal transition (EMT) is investigated.

Methods: 4NQO was painted on tongue of Swiss mice every third day at a dose of 25µl of 5mg/ml stock solution. After five consecutive treatment with 4NQO (starting Day7), one group of mice was treated with NLGP (s.c., 25µg/mice/week), keeping a group as PBS control. Mice were sacrificed in different time-intervals to harvest tongues and studied using histology, immunohistochemistry, flow-cytometry and RT-PCR on different immune cells and EMT markers (e-cadherin, vimentin) to elucidate their phenotypic and secretory status.

Results: Local administration of 4NQO for consecutive 300 days promotes significant alteration in tongue mucosa including erosion in papillae and migration of malignant epithelial cells to the underlying connective tissue stroma with the formation of cell nests (exophytic-hyperkeratosis with mild dysplasia). Therapeutic NLGP treatment delayed pre-neoplastic changes promoting normalization of mucosa by maintaining normal structure. Flow-cytometric evidences suggest that NLGP treatment upregulated $CD8^+$, $IFN\gamma^+$, granzyme B^+, $CD11c^+$ cells in comparison