

Evaluation of nicotinamide as an anti-inflammatory and anti-angiogenic agent in uveal melanoma cell lines

Avaliação da nicotinamida como agente anti-inflamatório e anti-angiogênico em linhas celulares de melanoma uveal

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ABSTRACT

Purpose: To investigate the effect of nicotinamide on the secretion of pro-angiogenic and pro-inflammatory cytokines in uveal melanoma cell lines.

Methods: Two human uveal melanoma cell lines (92.1 and OCM-1) were treated with nicotinamide (10 mmol/L) or control media for 48 hours in culture. The supernatant from each culture was used in sandwich enzyme-linked immunosorbent assay-based angiogenesis and inflammation arrays to evaluate the effects of exogenously administered nicotinamide on the secretion of a total of 20 pro-angiogenic and pro-inflammatory proteins.

Results: Seven pro-angiogenic cytokines were detected under control conditions for both uveal melanoma cell lines. Treatment with nicotinamide resulted in a significant decrease in secretion of the following pro-angiogenic cytokines: angiogenin, angiopoietin-2, epidermal growth factor, and vascular epithelial growth factor-A in the 92.1 cells; basic fibroblast growth factor in the OCM-1 cells; and placenta growth factor in both cell lines. Among the pro-inflammatory proteins, monocyte chemoattractant protein-1 and interleukin-8 were expressed in both untreated cell lines and both were significantly reduced when treated with nicotinamide.

Conclusions: Results from this *in vitro* model suggest that nicotinamide may have anti-inflammatory and anti-angiogenic properties, which may open the possibility of using it as a chemopreventive agent for uveal melanoma; however, further studies including animal models are warranted.

Keywords: Nicotinamide; Cytokines; Uveal neoplasms; Melanoma; Vascular endothelial growth factor A; Cell line

RESUMO

Objetivo: Acredita-se que a nicotinamida (NIC) seja capaz de diminuir a angiogênese induzida pelo fator de crescimento endotelial vascular (VEGF). Investigar os efeitos da nicotinamida sobre a secreção de citocinas pró-angiogênicas e pró-inflamatórias em linhagens de células de melanoma uveal humano (UM).

Métodos: Duas linhagens de células humanas de UM (92,1 e OCM-1) foram tratadas com NIC (10 mmol/L) ou apenas com meio de cultura por 48 horas. O sobrenadante das culturas obtido após a administração de nicotinamida foi comparado com o sobrenadante das culturas controle quanto à expressão de 20 fatores pró-angiogênicos e pró-inflamatórios, pela técnica de enzyme-linked immunosorbent assay (ELISA).

Resultados: Sete citocinas pró-angiogênicas foram detectadas nas condições de controle em ambas as linhagens de células de UM. O tratamento com nicotinamida promoveu uma redução significativa da secreção das seguintes citocinas angiogênicas: Angiogenina, ANG2, EGF e VEGF-A em células 92.1; bFGF em células OCM-1; PlGF em ambas as linhagens celulares. Quanto às proteínas pró-inflamatórias, a expressão de MCP-1 e IL-8 foi significativamente reduzida com a administração de nicotinamida em relação às culturas de células que não receberam o tratamento.

Conclusões: Nicotinamida apresenta propriedades anti-inflamatórias e anti-angiogênicas em modelo experimental *in vitro*. Tais efeitos sugerem a possibilidade de utilizar esta substância na quimioprevenção do UM. Entretanto, estudos com modelos experimentais *in vivo* são necessários para melhor avaliar o benefício do tratamento do UM com nicotinamida.

Descritores: Nicotinamida; Citocinas; Neoplasias uveais; Melanoma; Fator A de crescimento do endotélio vascular; Linhagem celular

INTRODUCTION

Uveal melanoma (UM) is a rare malignancy that accounts for 5.2% of all cutaneous and non-cutaneous melanomas⁽¹⁾, with a mean age-adjusted incidence in the United States of around 5.1 cases per million⁽²⁾. Most ocular melanomas are of uveal origin, with the most common of these arising from the choroid⁽¹⁾. UM is one of the most deadly cancers; approximately 50% of patients die from liver metastases⁽³⁾, and mortality has not changed over the last four decades despite many advances in treatment⁽²⁾.

Inflammation and angiogenesis play an important role in the pathogenesis of cancer; indeed, they are considered to be hallmarks of cancer formation and progression⁽⁴⁾. The cancer-related inflammatory milieu contains various inflammatory cells and pro-angiogenic and pro-inflammatory cytokines⁽⁴⁾. In UM, the presence of inflammatory cells and the degree of inflammation within the tumor correlate with poor prognosis⁽⁵⁾. Similar patterns are observed in breast cancer, where high tissue expression of vascular endothelial growth factor (VEGF)

and microvessel density are poor prognostic factors⁽⁶⁾, and in colorectal cancer, where systemic inflammation is associated with poor prognosis⁽⁷⁾.

Due to this close relationship between inflammation, angiogenesis, and cancer, various anti-angiogenic and anti-inflammatory agents have been shown to be beneficial in the treatment of cancer. One such agent is nicotinamide (NIC), the amide form of niacin (vitamin B3), which is a water-soluble vitamin involved in many cellular processes, especially adenosine triphosphate production. It has been suggested that NIC may show an inhibitory effect on angiogenesis by acting on various enzymes involved in the synthesis of pro-angiogenic factors such as sirtuins and poly (ADP-ribose) polymerase⁽⁸⁾. When keratinocytes are irradiated with ultraviolet-B radiation, NIC reduces various pro-inflammatory cytokines, such as interleukin (IL)-6, IL-10, monocyte chemoattractant protein (MCP)-1, and tumor necrosis factor (TNF) α ⁽⁹⁾. In human umbilical vein endothelial cells, NIC derivatives have been shown to reduce VEGF-induced angiogenesis⁽¹⁰⁾. Notably,

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NIC has been found to enhance the repair of irradiation-damaged DNA in normal skin melanocytes, suggesting it could be used for chemoprevention in skin melanoma⁽¹¹⁾.

These anti-angiogenic and anti-inflammatory properties of NIC make it a promising agent for cancer treatment or prevention⁽¹²⁾, but its effects on human UM have not yet been determined. In this study, therefore, we tested the anti-angiogenic and anti-inflammatory effects of NIC on two human UM cell lines to establish whether it has the potential to prevent or treat this high-mortality cancer.

METHODS

CELL CULTURES

Two human UM cell lines were used: 92.1 and OCM-1, initially established initially by Dr. Jager (at the University Hospital Leiden, The Netherlands) and Dr. Albert (University of Wisconsin-Madison, USA), respectively^(13,14). The cells were cultured in 75-cm² flasks at 37°C in a 5% CO₂-enriched atmosphere. The culture medium was GlutaMAX® (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (FBS; Invitrogen), 1% penicillin/streptomycin and 1% fungizone (Invitrogen).

MULTIPLEX SANDWICH ELISA

For each cell line, approximately 300,000 cells were seeded into all wells of a six-well plate. After 24 hours, the supernatant was discarded from all the wells, and serum-free media (SFM) was added to starve to cells for a further 24 hours. After a total of 48 hours, SFM with 10 mmol/L NIC (Sigma-Aldrich Corp., St. Louis, MO, USA) was added to three wells of the plate of each cell line. This concentration was chosen because it has been observed that the anti-inflammatory effect of NIC is dose-dependent between concentrations in blood of 2 to 40 mmol/L⁽¹⁵⁾; in addition, this concentration had been used previously in the cell culture by Audrito, et al.⁽¹⁶⁾. SFM without NIC was added to the remaining three wells of the plates for each cell line. After 48 hours, SFM with and without NIC for each cell line was collected into four tubes. Two different multiplex Quantibody® enzyme-linked immunosorbent assay (ELISA) arrays (QAH-ANG-1 and QAH-INF-1; RayBiotech Inc., Norcross, GA, USA) were run for both inflammation and angiogenesis.

ANGIOGENESIS ARRAY

The amounts of each of the various angiogenic factors produced by the cells were measured following the manufacturer’s protocol. Briefly, standard dilution curves were developed by diluting the lyophilized Cytokine Standard Mix to known concentrations of sample diluent provided in the Quantibody® Array Kit (RayBiotech). These diluted compounds were then applied to the array, whose glass surface contained bound antibodies specific to angiogenin, angiopoietin-2 (ANG-2), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), heparin-binding EGF-like growth factor (HB-EGF), hepatocyte growth factor (HGF), leptin, platelet-derived growth factor (PDGF-BB), placenta growth factor (PIGF), and VEGF. In addition, 100 µL from each of the four SFM tubes was applied to the array and, in a separate well, 100 µL of untreated SFM was applied to the array as a negative control. A second, biotinylated antibody was applied to the chambers of the slide and visualized with a laser scanner after adding a streptavidin-labeled fluorescent dye, thereby depicting fluorescence as a function of the concentration of pro-angiogenic factors. The array slide was then sent to the company (RayBiotech) for analysis.

INFLAMMATION ARRAY

A similar Quantibody® array was run for pro-inflammatory cytokines, including IL-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-13, MCP-1, interferon-delta (INFδ), and TNFα. The procedure was identical to that described for the angiogenesis array.

STATISTICAL ANALYSIS

We used Microsoft Excel software (Microsoft Office® 2011; Microsoft Corp., Redmond, WA, USA) to analyze the data. Comparisons used a two-tailed, unpaired Student’s t-test, with P values less than 0.05 considered statistically significant.

RESULTS

Angiogenesis array: Seven pro-angiogenic factors (angiogenin, ANG-2, EGF, bFGF, HB-EGF, PIGF, and VEGF) were detected in the control samples for both UM cell lines. Following treatment with NIC, the OCM-1 cells showed a significant decrease in bFGF and PIGF levels. In the 92.1 cells treated with NIC, angiogenin, ANG-2, EGF, PIGF, and VEGF levels were significantly reduced (Table 1).

Inflammatory array: In both cell lines, only IL-8 and MCP-1 were detected under the control conditions. After treatment with NIC, both cell lines showed significantly lower levels of IL-8 and MCP-1 production (Table 2).

Table 1. The angiogenesis array: concentrations (in pg/mL) of the angiogenic factors secreted by OCM-1 and 92.1 uveal melanoma cell lines with or without treatment with nicotinamide

Factor	OCM 1 + control	OCM 1 + NIC	P value	92.1 + control	92.1 + NIC	P value
Angiogenin	5701.5	5258.0	0.05	6966.1	5669.4*	<0.05
ANG-2	667.0	632.3	0.78	766.7	641.5*	<0.05
EGF	3.8	3.9	0.61	10.3	6.9*	<0.05
bFGF	1459.5	88.1*	<0.05	282.9	275.6	0.31
HB-EGF	1060.4	849.9	0.10	1156.3	1001.9	0.11
HGF	ND	ND		ND	ND	
Leptin	ND	ND		ND	ND	
PDGF-BB	ND	ND		ND	ND	
PIGF	61.9	23.5*	<0.05	199.3	132.0*	<0.05
VEGF	17698.3	15588.9	0.11	27494.3	24814.7*	<0.05

*= values with statistical significance at P<0.05. Values in gray= below the limit of detection; ND= not detected; NIC= nicotinamide; ANG-2= angiopoietin-2; EGF= epidermal growth factor; bFGF= basic fibroblast growth factor; HB-EGF= heparin-binding EGF-like growth factor; HGF= hepatocyte growth factor; PDGF-BB= platelet-derived growth factor; PIGF= placenta growth factor; VEGF= vascular endothelial growth factor.

Table 2. The inflammatory array: concentrations (in pg/mL) of the inflammatory factors secreted by OCM-1 and 92.1 uveal melanoma cell lines with or without treatment with nicotinamide

Factor	OCM 1 + control	OCM 1 + NIC	P value	92.1 + control	92.1 + NIC	P value
IL-1a	ND	0.9	0.16	ND	0.6	0.14
IL-1b	ND	ND		ND	ND	
IL-4	ND	ND		ND	ND	
IL-6	0.3	1.3	0.01	ND	1.2	0.00
IL-8	9.4	3.6*	<0.05	11.1	6.4*	<0.05
IL-10	ND	ND		ND	ND	
IL-13	0.8	5.9	0.01	ND	3.5	0.00
MCP-1	40.6	20.3*	<0.05	80.7	50.3*	<0.05
INFδ	0.5	2	0.13	ND	2.1	0.00
TNFα	3.1	4.8*	0.03	ND	2.7	0.00

*= values with statistical significance at P<0.05. Values in gray= below the limit of detection. ND= not detected; NIC= nicotinamide; IL= interleukin; MCP= monocyte chemotactic protein; INF= interferon; TNF= tumor necrosis factor.

DISCUSSION

Because inflammation and angiogenesis are closely linked to the progression of melanoma⁽¹⁷⁾, we evaluated the levels of various pro-angiogenic and pro-inflammatory cytokines secreted by two human UM cell lines after NIC treatment. Both showed a significant decrease in cytokine levels after treatment with 10 mmol/L NIC for 48 hours.

In the angiogenesis assay, we found that both VEGF and EGF significantly decreased in the 92.1 cell line after NIC treatment. A meta-analysis⁽¹⁸⁾ showed VEGF expression to be significantly higher in UM patients than in matched controls; in addition, VEGF expression in tumors was higher in patients with poor prognostic factors such as age <50 years, scleral invasion, large tumors, and predominantly epithelioid cell-type tumors. Conversely, EGF has been shown to raise transforming growth factor- β and to promote the epithelial-mesenchymal transition that leads to metastasis⁽¹⁹⁾. Furthermore, targeting angiogenin has been found to reduce tumorigenesis and the metastasis of bladder cancer cells in nude mice⁽²⁰⁾. In the present study, angiogenin decreased after NIC treatment in the 92.1 cell line, as did ANG-2, which shows great potential for anti-angiogenic therapy⁽²¹⁾. Interestingly, when the OCM-1 cell line was treated with NIC, there was no significant decrease in VEGF or angiogenin, although near-significant reductions were observed. However, in the OCM-1 cells, NIC significantly downregulated bFGF, which is a factor of poor prognosis in breast cancer⁽²²⁾.

PIGF has been extensively studied and is currently being used in targeted therapy for various cancers, including medulloblastoma⁽²³⁾. Remarkably, both of our cell lines showed significant decreases in this pro-angiogenic factor after NIC treatment. In addition, both cell lines showed somewhat lower HB-EGF levels after treatment, although these results were not statistically significant. HB-EGF is known to be a potent inducer of tumor growth and angiogenesis⁽²⁴⁾, and the HB-EGF pathway has recently been targeted as an alternative cancer treatment⁽²⁵⁾.

The effect of NIC on inflammation is controversial. In an endotoxemia model, a concentration of 4 mmol/L NIC in human serum reduced the level only of IL-6, whereas at 40 mmol/L the levels of IL-1 β , IL-6, and TNF α were significantly reduced⁽¹⁵⁾. However, when healthy volunteers were treated with endotoxin, even a high oral dose of NIC did not affect the levels of pro-inflammatory cytokines⁽²⁶⁾. This could be a limitation of the clinical application of NIC, or it may indicate the need for other methods of administration; further studies of this are needed with regard to UM and NIC.

Tumor-associated macrophages have previously been studied in cutaneous melanoma⁽²⁷⁾ and UM⁽²⁸⁾. They are necessary for angiogenesis as well as invasion, and they have been shown to have a strong correlation with mortality from metastases⁽²⁹⁾. In tumors, cytokines are responsible for macrophage recruitment and differentiation. In the present study, MCP-1, which is known to play a key role as a chemokine⁽²⁹⁾, decreased in our treated cells.

It has been shown that the UM microenvironment can activate IL-8 signaling, thereby promoting angiogenesis⁽³⁰⁾. As our results showed that NIC downregulates IL-8, applying NIC in an *in vivo* setting may reduce the angiogenesis required for UM growth. Furthermore, as the foremost prognostic indicator for UM is the largest tumor dimension, early treatment with NIC may have a profound effect on UM metastasis.

In chronic lymphocytic leukemia, it has been shown that, in culture, exposure to NIC was followed by a significant decrease in the enzymatic activity of sirtuin-1 (SIRT1)⁽¹⁶⁾. Although we did not investigate the effects of NIC on SIRT1 in the present study, this is an interesting pathway that warrants further research as the majority of UM tumors express SIRT1⁽³¹⁾. Patients with UM tumors negative for SIRT1 expression have been observed to develop metastasis at a greater rate than those exhibiting SIRT1 expression, in contrast to the expression of SIRT1 in other cancers, in which metastatic disease correlates with the upregulation of this deacetylase⁽³¹⁾.

There were limitations to this study. For instance, we only studied two cell lines, which may not be representative of the entire disease spectrum. However, the two cell lines we chose are located at both ends of the malignant spectrum; one (92.1) has high metastatic and proliferative potential and the other (OCM-1) has low metastatic and proliferative potential. Results cannot be compared between the OCM-1 and 92.1 cell lines because of these different proliferation rates and metastatic potentials.

It is important to recognize that the present study only evaluated the secretion of pro-angiogenic and pro-inflammatory cytokines, and did not examine transcriptional changes in these proteins. The reason for this is that it has been well established that intercellular signaling is a key driver of angiogenic processes and tumor invasion in UM and other cancers. Primary UM is generally well diagnosed and managed clinically, and so the focus of this investigation was on processes that promote the establishment and growth of metastatic tumors.

Now that these results have demonstrated the effects of NIC on pro-angiogenic and inflammatory cytokines *in vitro*, further investigation should test different doses to determine the optimal doses for *in vivo* models. In addition, the effects of NIC on cell proliferation and viability are unreliable because an *in vitro* angiogenic model is not yet available. The clinical utility of NIC should also be tested in UM animal models.

In conclusion, treatment with NIC resulted in a significant reduction in the levels of several pro-angiogenic cytokines in two human UM cell lines. NIC treatment also significantly reduced MCP-1 and IL-8 levels. These findings suggest that NIC has both anti-angiogenic and anti-inflammatory effects on UM cells, and provides the basis for further studies and the possible use of NIC as a therapeutic agent in UM.

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