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ORIGINAL ARTICLE

Intranasal Administration of Type V Collagen Reduces Lung Carcinogenesis through Increasing Endothelial and Epithelial Apoptosis in a Urethane-Induced Lung Tumor Model

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Abstract Type V collagen (Col V) is a "minor" component of normal lung extracellular matrix, which is subjected to decreased and abnormal synthesis in human lung infiltrating adenocarcinoma. We previously reported that a direct link between low amounts of Col V and decreased cell apoptosis may favor cancer cell growth in the mouse lung after chemical carcinogenesis. Moreover, this collagen species was able to trigger DNA fragmentation and impair survival of neoplastic cells. In this study, we have extended our investigation with the aim to obtain further evidence that the death induced by Col V-treatment is of the caspase-9 apoptotic type. We used (1) optical and electron microscopy, (2) quantitation of TUNEL-labeled cells and (3) analysis of the expression levels of Col V and selected genes coding for apoptosis-linked factors, by conventional RT-PCR. BALB/c mice were injected intraperitoneally with 1.5 g/kg body weight of urethane. After urethane injection, the animals received intranasal administration of 20 μg/20 μl of Col V every day during 2 months. We report here that Col V treatment was able to determine significant increase in Col V protein and gene expression and in the percentage of TUNEL-positive cells, to up-regulate caspase-9, resulting in low growth of tumor cells. Our data validate chemical carcinogenesis as a suitable "in vivo" model for further and more detailed studies on the molecular mechanisms of the death response induced by Col V in lung infiltrating adenocarcinoma opening new strategies for treatment.

Keywords Chemically-induced lung carcinogenesis · Molecular biology · Apoptosis · Type V collagen · Immune response · Vascular endothelial growth factor

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Introduction

Lung cancer remains the major cause of death worldwide (Jemal et al. 2011). While in the last 20 years the death rate for neoplastic diseases has decreased in developed countries lung cancer prevalence has been increasing (Jemal et al. 2011). The 5-year survival rate of lung cancer is modestly 16.3 % (2015). This aggressiveness may be mainly due to its invasive properties by substantial degradation of the basement membrane and significant changes of the interstitial collagen component of the extracellular matrix.

The type V collagen (Col V), a minor collagen of normal lung, is intercalated within fibrils of type I collagen, the major lung collagen (Linsenmayer et al. 1993). Recent results provided evidence that Col V is subjected to decreased and abnormal synthesis in human lung infiltrating adenocarcinoma (Souza et al. 2010), thus acting as a poorly-adhesive substrate and facilitating motility and invasion of the neoplastic cells (Luparello 1994; Luparello et al. 1990; Minafra et al. 1992). On the other side, adhesion of neoplastic cells onto Col V resulted in a prominent decrease of growth rate and inhibition of motility and invasion ''in vitro''(Luparello and Sirchia 2005). Interestingly, previous work done by Luparello et al. (2001) suggest that the growth-restraining activity shown by Col V on neoplastic cells could be ascribed to an increase of cell

death rate. In fact, our group reported that a direct link between low amounts of Col V and decreased cell apoptosis enhanced cancer cell growth in the mouse lung after urethane carcinogenesis (Parra et al. 2010). In light of these reports suggesting correlation between Col V and neoplasic cell apoptosis, we extended our investigation with the aim to (1) obtain further evidence by a specific assay that the Col V-induced death was of the apoptotic type, (2) determine whether this collagen substrate could exert some effect on the expression level of genes coding for apoptosis regulators and response proteins (Hanahan and Weinberg 2011; Saraste and Pulkki 2000). We expect that the results of this study may help to detail molecular mechanisms and opening new strategies for treatment.

Materials and Methods

Animals

Specific pathogen-free male BALB/c mice were used. The animals were maintained at 23 °C under a 12-h light–dark cycle and were allowed access to food and water *ad libitum*. This study was carried out in strict accordance with the principles and guidelines adopted by the Brazilian College of Animal Experimentation and approved by the Ethical Committee for Animal Research of the School of Medicine, University of Sao Paulo (Protocol No.: 0960/08).

Preparation of Col V

Col V was isolated from human placenta as previously described (Bezerra et al. 2006; Teodoro et al. 2003, 2004). The lyophilized Col V was solubilized in 0.01 N acetic acid at final concentration of 1 mg/ml. The preparation was dialyzed in distilled water during 48 h.

Urethane-Induced Lung Carcinogenesis (U)

Twenty male BALB/c mice were treated with 3 g/kg body weight of urethane provided by the pharmacy of our teaching hospital. The drug was administered in two intraperitoneal (i.p.) injections of 1.5 g/kg, with 48-h of interval. A previous study showing this dose to be the highest concentration that did not elicit massive mortality immediately after injection and that promoted the development of identifiable pulmonary nodules within a time span of 48 days (Cury et al. 2000; Reymao et al. 1997). Ten male BALB/c mice were not exposed to urethane and composed a control group.

Col V Therapy by Intranasal Administration

Mice were inoculated with urethane as described above and were randomly allocated to two groups of ten animals. On day 60 after the first urethane inoculation, one group received intranasal (i.n.) administration of Col V (20 μg/20 μl), every day during 2 months (U-COL V group). The other urethane induced mice group did not receive Col V, but only the same volume of the vehicle of dialyzed acetic acid 0.01 N (U group). Control animals ($n = 20$) received Col V (20 μg/20 μl), every day during 2 months (COL V group), or vehicle alone (control group), according to other authors (Yasufuku et al. 2001). This dose of collagen was chosen because of its effectiveness determined on previous experiments (unpublished data) using 20–40 μg/20 μl, according to other studies done by our group and other investigators (Garippo et al. 2007; Lott et al. 2013; Velosa et al. 2007, 2010; Yasufuku et al. 2001; Yoshino et al. 1995).

Tissue Preparation

All the animals were killed at the end of the intranasal administration of Col V period. The mice were sedated, anesthetized with 20 mg/kg i.p. pentobarbital sodium and exsanguinated by sectioning the caudal aorta. The lungs were rapidly removed and divided to the histological study and to genetic study. The lungs to the histological study were dissected and rinsed free of blood with saline solution, inflated *in situ* through the bronchiole at a pressure of 15 mm H_2O, measured with respect to tidal volume and fixed with 10 ml/kg (0.2 ml) of 10 % neutral buffered formalin for 6 h to complete tissue fixation and embedded in paraffin. Thin sections were stained with hematoxylin and eosin (H&E). Additional serial sections from all the paraffin blocks were used for immunohistochemistry and immunofluorescence. The tumors designated to the molecular biology analysis were immediately inserted into criogenic tubes and stored at liquid nitrogen. Finally, after fixation in glutaraldehyde for 2 h and transferred to a saline solution few tumors were analyzed through electron microscopy.

RNA Extraction

Tissue specimens were pulverized (Bio-Pulverizer-BioSpec Products Inc., Bartlesville, OK, USA) under liquid nitrogen and total mouse RNA was extracted from approximately 100 mg of tissue after homogenization using the Trizol reagent (Invitrogen life technology, Scotland, UK) according to the manufacturer's instructions.

Before proceeding with the RT-PCR, the extracted RNA quality and integrity was verified through spectrophotometry (absorbance at 260:280 nm—A260/280) and through observation of 18S/28S rRNA on 1 % agarose gel electrophoresis under denaturing conditions and visualized with ethidium bromide (ratio: >1.0).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

cDNA synthesis was performed using 4 µg of total RNA and SuperScript First-Strand Synthesis System for RT-PCR Kit (Invitrogen life technology, Scotland, UK). Aliquots (5 µl) of cDNA were added to a 20 µl reaction mixture containing 20 µl of 2 × SYBR Green PCR Master Mix (Invitrogen life technology, Scotland, UK) and 0.3 mM of each primer for triplicate reactions in a 20 µl volume per well. Triplicate reactions were performed and averaged. The reaction was first incubated at 95 °C for 15 s, followed by 40 cycles of annealing at 56 °C for 60 s and extension at 72 °C for 1 min. Primer sets were designed based on the coding region closer to the 3′ end of the gene using primers. The primer sequences for the RT-PCR are shown in Table 1.

Reactions were conducted on the StepOnePlus (Applied Biosystems, San Diego, CA, USA). The data analysis was done through the StepOne software v2.0 (Applied Biosystems, San Diego, CAa, USA). Results displaying a CT intra-variation <1.5 were further used to calculate average values. Data were expressed as CT values (the cycle number at which logarithmic PCR plots cross a calculated threshold line). Relative expression of genes of interest was normalized to that of GAPDH and gene expression in each sample was then compared with the expression in pool cells. The comparative CT method (CT) was used for quantification of gene expression and the relative expression was calculated as 2 CT.

Table 1 Primers used for quantitative real-time PCR

Type V collagen	
Primer 1	GCTCCAACGATGAGGAAATG
Primer 2	GCCAAAGTCGTTGAACATGA
CASPASE 9	
Primer 1	AATGGGACTCACAGCAAGG
Primer 2	GGGACTGCAGGTATTCAGAG
VEGF isoforms	
Primer 1	GGAGAGATGAGCTTCCTACAGCA
Primer 2 (VEGF 120)	CTGAACAAGGCTCACAGTGATTTT
Primer 2 (VEGF 164)	CCTCGGCTTGTCACATTTTTCT
Primer 2 (VEGF 188)	GCTCACAGTGATTTTCTGGCTT

Immunohistochemistry

The antibodies used were caspase-9 (Temecula, CA, USA; dilution 1:6000) that is specific for the cleaved form of caspase-9. The Novolink Max Polymer (Novocastra Laboratories LTDA), pressure-cooking antigen retrieval, biotinylated rabbit antimouse IgG (Dako Corp.; dilution 1:400), and streptavidin were used in combination with biotinylated horseradish peroxidase (Dako Corp.; dilution 1:1000), diaminobenzidine tetrahydrochloride, and counterstaining with Hematoxylin. Brownish cytoplasmic staining was considered evidence of antigen expression by the cells.

Optical Microscopy

Tissue specimens were selected from each group, processed for paraffin embedding, sectioned (three microns) and stained with H&E for light microscopy, using a Nikon microscope. The tumors were submitted to qualitative and quantitative histopathological analysis. They were classified according to pathological criteria proposed by Kauffman et al. (1979) and Ward et al. (1985). Two pathologists (ERP and VLC), blinded to the groups' assignment performed the classification.

Electron Microscopy

Tumoral tissues were fixed in 2.5 % glutaraldehyde in phosphate buffer (0.1 mol/L, pH 7.4) for at least 2 h at 48 °C. The tissue sections were secondarily fixed in 1 % osmium tetroxide and 0.8 % potassium ferrocyanide for 1 h at 48 °C. After three washes with cold double distilled water, the samples were dehydrated with ascending concentrations of acetone (30, 50, 70, 95 and 100 %), and three changes of 100 % acetone. They were then embedded in Spurr resin and polymerized at 608 °C. The embedded blocks were sectioned with a diamond knife (Diatome, Biel, Switzerland) in a Leica ultramicrotome (Leica Microsystems, Deerfield, IL, USA). Ultrathin sections were placed on copper grids and stained with uranyl acetate and lead citrate before examination with a JEM 1010 microscope (JEOL Ltd., Tokyo, Japan) equipped with a Gatan/BioScan digital camera (Gatan Inc., Pleasanton, CA, USA).

In situ Detection of Apoptotic Cells

For the in situ detection of apoptosis at the level of a single cell, we used an apoptotic assay of the deoxynucleotidyl transferase (TdT) method of end labeling (TUNEL) (Boehringer Mannheim, Germany) (Gavrieli et al. 1992; Wijsman et al. 1993). Paraffin wax-embedded sections (3–4 µm) were layered onto glass slides. The tissue

sections were dewaxed with xylene and rehydrated with graded dilutions of ethanol in water. The slides were then washed four times with double-distilled water for two minutes and immersed in TdT buffer (Boehringer Mannheim). The sections were then covered with TdT (0.3 U/μl) and fluorescein-labeled dUTP in TdT buffer, and the samples were incubated in a humid atmosphere at 37 °C for 60 min. For negative controls, TdT was eliminated from the reaction mixture. The sections were then incubated with a peroxidase-conjugated antibody specific for fluorescein. The staining was observed with a substrate system in which nuclei with DNA fragmentation were stained brown. The reaction was terminated by washing the sections twice in phosphate-buffered saline. Nuclei without DNA fragmentation were stained blue as a result of counterstaining with Hematoxylin.

Immunofluorescence

Antigen retrieval was carried out by the enzymatic treatment of lungs with bovine pepsin (10,000 U/ml) (Sigma Diagnostics, St. Louis, MO, USA) 4 mg/ml in acid acetic 0.5 N incubated for 30 min at 37 °C. Then, the slips were incubated overnight with rabbit polyclonal antihuman Col V antibody (1:2000, Col V from human placenta; Sigma Chemical Co., Saint Louis, MO, USA), raised in our laboratory according to previous works of Miller and Rhodes (1982). For negative controls, sections were incubated with fetal bovine serum instead of the primary antibody. The same tissue treatment was used for the immunofluorescence detection. The sections were incubated with the same primary antibody, diluted with PBS, plus 1 % bovine serum albumin overnight at 4 °C in a humid atmosphere. The sections were then incubated with a Fluorescein Isothiocyanate (FITC)-conjugate goat anti-rabbit immunoglobulin (Sigma Chemical Co.; dilution 1:50) as a secondary antibody, and mounted with an aqueous mounting medium. Col V fibers were quantified in the lung ECM from the U, U-COL V and Control groups by image analysis. Briefly, an image analysis system consisting of a Nikon camera mounted on a Nikon microscope sent the images to a LG monitor by means of a computer-controlled (Pentium 1330 MHz) digitizing system (Oculus TCX, Coreco Inc., St. Laurent, Quebec, Canada). Images were processed by Image-Pro Plus 7.0 software (Media Cybernetics, Inc., Bethesda, MD, USA). For each lung specimen, the ECM compartment in the tumor, surround tumor and ten normal samples of ECM from control group were analyzed at a magnification of ×400. The collagen fibers densities in this compartment were expressed as the amount of fibers divided by the total area studied. The final results are expressed as the number of collagen fibers per total area (Hsia et al. 2010).

Histomorphometry

The area fraction occupied by the tumor in the lung parenchyma and the apoptotic caspase-9^+ and $TUNEL^+$ epithelial and endothelial cells in lungs from the all groups were evaluated by stereology. At ×400 magnification, an eyepiece systematic point-sampling grid with 100 points and 50 lines was used to count the number of points overlying positively-stained cells in the cytoplasm (caspase-9) and nucleus (TUNEL), respectively (Hsia et al. 2010). To evaluate tumor growth, the same procedure was applied to count the number of points overlying tumors from the Cancer and Col V groups. Measurements were averaged over ten microscopic fields to obtain the apoptotic and tumor growth index percentages.

Comparisons were performed in 20 % of the slides by two observers (ERP and VLC). The coefficient of variation for the inter-observer error regarding cell count was <5 %.

Statistical Analysis

Data are expressed as the mean (standard error of the mean) with 95 % confidence intervals. Statistical analysis was performed by analysis of variance, followed by appropriate *post hoc* tests, including Bonferroni for multiple comparisons by the one-way analysis of variance (ANOVA) test and Student's t test for two variables between groups. For verification of association between the different genes expression we used Bivariate Pearson's test. The statistical program used was SPSS 18.0 (SPSS Inc., Chicago, IL, USA). A $p < 0.05$ was considered significant.

Results

Lung parenchyma from control, urethane and urethane plus Col V animals are shown in Fig. 1 stained by H&E (A, F, K), immunofluorescence for Col V (B, G, L), immunohistochemistry for caspase-9 (C,H,M), TUNEL for *in situ* apoptosis detection (D, I, N) and electron microscopy for apoptosis (E, J, O).

Control animals show normal alveoli (A), a green birefringence of Col V fibers observed under fluorescent microscopy (B), which are organized in a subtle fibrillary pattern along of alveolar walls (B), scant tumor cells in apoptosis by caspase-9 (C) or TUNEL (D) detection. Electron microscopy shows details of normal alveoli type 2 pneumocytes covering the alveolar space and separated of the capillary by basal membrane and thin layer containing collagen fibers (E). In non-explicit apoptosis, type II pneumocytes have fine and uniformly dispersed chromatin within the nuclear membrane (E).

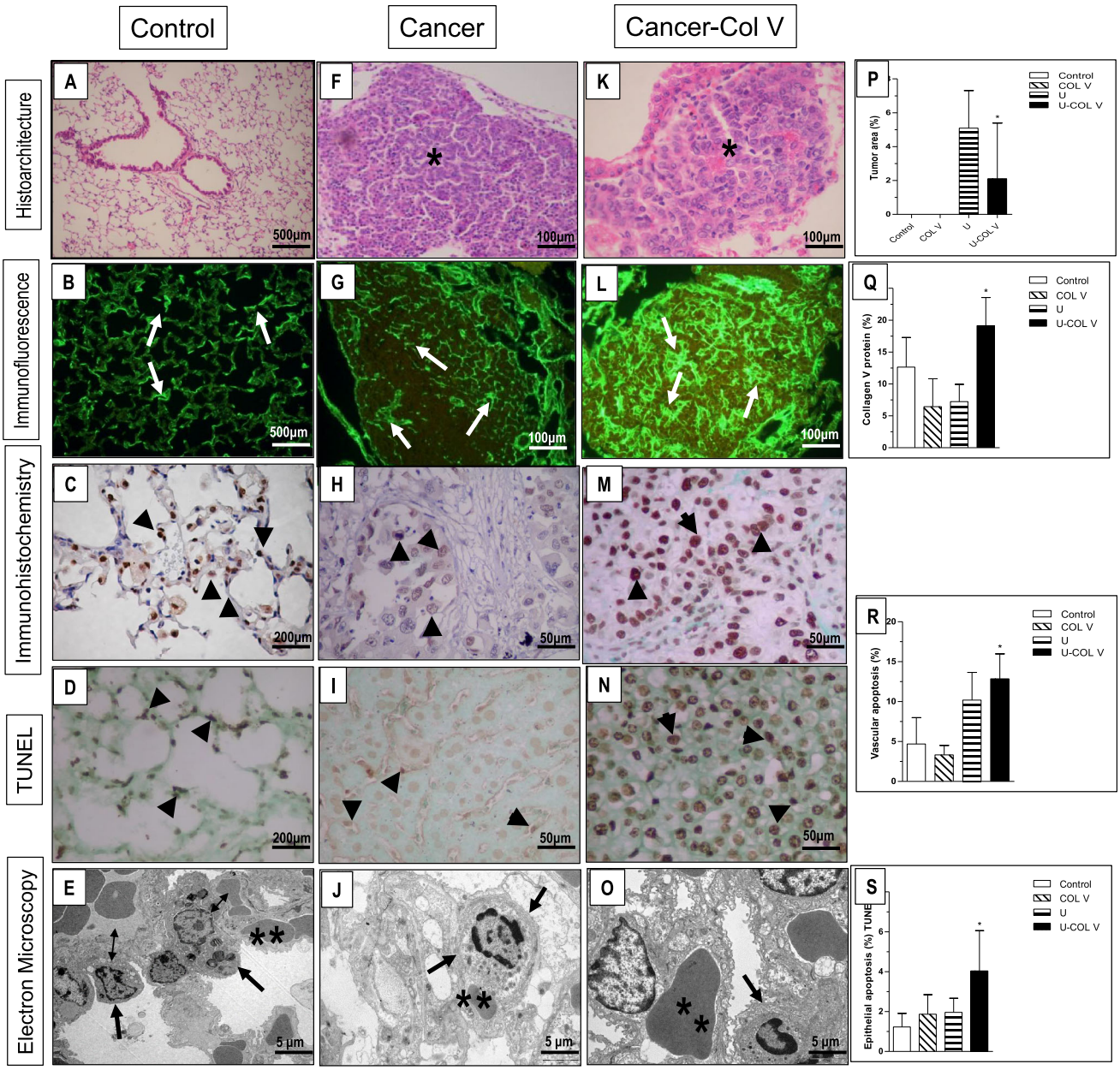

Fig. 1 Lung parenchyma from control, urethane and urethane plus Col V animals stained by H&E (**a**, **f**, **k**), immunofluorescence for Col V (**b**, **g**, **l**), immunohistochemistry for caspase-9 (**c**, **h**, **m**), TUNEL for in situ apoptosis detection (**d**, **i**, **n**) and electron microscopy for apoptosis (**e**, **j**, **o**). Control animals show normal alveoli (**a**), a green birefringence of Col V fibrillary pattern along of alveolar walls (**b**), scant tumor cells in apoptosis by caspase-9 (**c**) or TUNEL (**d**) detection and type II pneumocytes with fine and uniformly dispersed chromatin within the nuclear membrane (**e**). Urethane animals exhibit epithelial tumors (**f**) with decreased fibrillary pattern of Col V around individual malignant cells and vessels (**g**), discrete number of tumor cells in apoptosis caspase-9 (**h**) and TUNEL positive (**i**); note the nuclei of malignant cells showing marginated masses of chromatin typical pathological features of apoptosis (**j**) and the fibrillary disarrangement of collagen fibers (**j**). Compared to control and urethane animals, epithelial tumors in urethane plus Col V animals present significant reduction of the area fraction (**k**, **p**), a prominent texture of thin Col V fibers (**l**, **q**) coincident with the H&E phenotype (**k**) and significant increase of tumor cells in apoptosis caspase-9 (**m**, **r**) or TUNEL (**n**, **r**) positive; note that malignant cells exhibit condensed chromatin close to the nuclear membrane, characteristic of tumor (**m**, **r**) and endothelial cells (**o**, **s**) in apoptosis, with less collagen fibers represented by amorphous than fibrillary matrix (**o**). *Arrowheads* indicate caspase-9 and TUNEL apoptosis; *arrows* indicate type 2 pneumocytes; *single asterisk* epithelial tumor and *double asterisks* indicate epithelial/endothelial basement membrane. *H&E* hematoxylin and eosin, *Col V* type V collagen, *U* urethane, *U-Col V* urethane plus Col V

Urethane animals exhibit substitution of alveoli by epithelial tumors (F), with the green birefringence of Col V organized in an irregular fibrillary pattern around individual malignant cells, as well as those around new vessels of the tumor microenvironment (G). In this group, discrete number of tumor cells expresses apoptosis caspase-9 (H) and TUNEL positive (I). The detection of apoptotic cells by in situ end labelling of fragmented DNA was supported by the fact that pathological criteria for apoptosis were also evident on electron and light microscopy. In fact, in sections stained with H&E, cytologically malignant cells exhibit hyperchromatic nuclei (F). By electron microscopy, the nuclei of malignant cells showed marginated masses of chromatin, along with discrete, well preserved apoptotic nuclei—typical pathological features of apoptosis (J). The fibrillar disarrangement of collagen fibers (J) is observed in the tumor microenviroment.

In contrast, epithelial tumors in urethane plus Col V animals presented reduction of the area fraction (K), Col V fibers organized in a prominent texture of thin fibers consisting of a complex glandular proliferation in tumor microenvironment (L) coincident with the H&E phenotype (K). In this group, more tumor cells in apoptosis caspase-9 (M) or TUNEL (N) were detected. By electron microscopy we observed cytologically malignant cells exhibiting condensed chromatin close to the nuclear membrane thus characterizing tumor (O) and endothelial cells (O) apoptosis, with less collagen fibers in the tumor microenvironment (O). The tumor peripheral microenvironment in this group is more represented by amorphous than fibrillary matrix (O).

Graphic illustration of measurements from control, urethane and urethane plus Col V animals are shown in Fig. 1 (panel P, Q, R, S). Table 2 summarizes the morphometric results for tumor area, endothelial and epithelial apoptosis and Col V fibers. A significant reduction of tumor area fraction was found in urethane plus Col V compared to urethane animals (P). The level of Col V is significantly higher in urethane plus Col V tumors compared to urethane tumors (Q). A significant difference of endothelium caspase-9^{+} apoptosis index is found in both animal groups (R). Also significant was the epithelial TUNEL apoptosis index in tumors from urethane and urethane plus Col V animals (S). In addition, according to a univariate analysis, Col V was positively related to the amount of endothelium apoptosis caspase-9^{+}. In other words, lower levels of tumor with Col V were associated with low fraction of endothelium apoptosis caspase-9^{+}.

Table 3 summarizes the Col V, VEGF 120, VEGF 164 VEGF 188 and caspase-9 mRNA expression gene levels. A

Table 2 Summary of morphometric results

Variable	Control	Col V	Cancer (U)	Urethane plus Col V	*p* value
Tumor area (%)	Absent	Absent	5.09 ± 2.22	2.11 ± 3.28[a]	0.02
Endothelium apoptosis (%)	4.68 ± 3.31	2.80 ± 0.98	10.18 ± 3.47	12.85 ± 3.12[a]	0.01
Epithelial apoptosis (%)	1.23 ± 0.68	2.25 ± 0.74	1.96 ± 0.71	4.04 ± 2.02[a]	0.05
Col V (%)	1.64 ± 0.46	5.44 ± 2.41	7.23 ± 2.71	19.15 ± 4.40[a]	0.01

Values are means (±SD) of 10 animals in each group. All values were computed in ten random, non-coincident fields per mice. Fraction area of tumor area, endothelium apoptosis caspase-9^{+}, epithelial apoptosis TUNEL^{+} and Col V. In control group, vehicle (dialyzed acetic acid 0.01 N) was intranasal instilled. In cancer group, mice received two i.p. injections of 1.5 g/kg, with 48-h of interval. At day 60, urethane plus Col V received intranasal administration of Col V (20 μg/20 μl), every day during 2 months

[a] Cancer vs control group. Col V: type V collagen

Table 3 Summary of Col V, VEGF 120, VEGF 164 VEGF 188 and caspase-9 genes expression, stratified by control, Col V, U and U-Col V groups

Gene expression	Control	Col V	Cancer (U)	Urethane plus Col V	*p* value
Col V	0.72 ± 0.62	0.98 ± 0.05	0.31 ± 0.39	1.92 ± 0.61*	0.001
VEGF-120	0.89 ± 0.55	0.77 ± 0.03	1.06 ± 0.69	1.10 ± 0.47	>0.05
VEGF-164	0.85 ± 0.52	0.80 ± 0.04	1.50 ± 1.66	0.86 ± 0.34	>0.05
VEGF-188	1.31 ± 0.65	0.97 ± 0.02	1.52 ± 1.46	0.74 ± 0.66	>0.05
Caspase-9	0.001 ± 0.0008	1.04 ± 0.03	0.001 ± 0.002	0.22 ± 023*	0.03

Table shows qRT-PCR results and numbers represent relative gene expression normalized by GAPDH

* Significantly different between urethane plus Col V and cancer or control groups

significant higher expression of Col V mRNA expression is observed between urethane plus Col V and control groups. Equally significant is the higher caspase-9 mRNA gene expression in urethane plus Col V compared to control groups. No statistical difference was found for different VEGF mRNA expression gene in the groups analyzed.

Discussion

In this study we assessed the modulatory effects of anti-Col V immunity induced by nasal administration of Col V on the stromal collagen and DNA genomic damage induced by urethane carcinogenesis through electron microscopy, TUNEL, immunohistochemistry, and molecular biology assays. We expanded previous analyses reported by Parra et al. (2010), and determined that anti-Col V immunity induced by nasal administration of Col V impairs survival of malignant cells by urethane promoting a caspase-dependent apoptosis, providing also some information at gene expression level. We also showed that the high amount of Col V fibers deposition after nasal administration of Col V on the stroma may endorses a direct binding to stromal receptors resulting in a prominent slowing of growth rate, inhibition of motility and invasion. Further investigations should be done to find the specific intracellular pathway through which Col V type achieves its death action on cancer cells; nevertheless, our data here described permit to make some comments.

Urethane or ethyl carbamate is a chemical carcinogen present in tobacco leaves, tobacco smoke (Enomoto et al. 2008) and in fermented food products (Patrignani et al. 2012) formed by the reaction of urea with alcohol (Schehl et al. 2007). Urethane metabolites cause oxidative stress in DNA molecules, developing adducts and involving C-hydroxylation to form vinyl carbamate, which is then converted to an epoxide that can interact with nucleic acid (Zimmerli and Schlatter 1991). The resulting reactive oxygen and nitrogen species play an important role in the initiation, promotion, and progression stages of the malignant transformation (Zimmerli and Schlatter 1991). The next sequence of events involves cancer cells quantitative apoptosis and changes in the stromal collagen component induced either by tumoral and endothelial cells, which facilitate the adhesion and migration of malignant cells and the penetration of the tumor by blood vessels.

In the current study, histologic examination of lungs from animals that received i.p. urethane, after 60 days revealed malignant neoplastic cells infiltrating a fibrillary stroma. In contrast, animals treated with nasal administration of Col V presented significant reduction of fraction area occupied by malignant neoplastic cells. Lung investigation of these animals treated with nasal administration of Col V by immunofluorescence and molecular assays revealed that the Col V fibers were significantly increased in tumor fibrillary stroma coinciding with increased mRNA expression of the (α1 Col V) chain from the Col V gene also indicating increased Col V synthesis in lung tumor. This finding achieves support in the literature showing that the high amount of Col V present in breast cancer promotes a direct binding to a non-integrin receptor resulting in a prominent stop of growth rate, difficult motility and invasion. Taken together, these results suggest that genes that code for increased mRNA expression of the (α1 Col V) may play an important role in the regression of lung tumors. Interesting enough, is the current statement that tumor stromal remodeling may result in Col V exposure and generation of anti-ColV immune response in a similar way reported in diseases associated with lung remodeling (Braun et al. 2010; Garippo et al. 2007; Velosa et al. 2010) and lung allograft rejection (Braun et al. 2009; Yasufuku et al. 2001, 2002). These data sustain the hypothesis that anti-Col V immunity induced by nasal administration of Col V may limit the lung tumor progression.

At this point it is important to make a brief comment about the different cellular response recruited by this immunogenic collagen, which will depend on cytotype involved in the process. In fact, Col V may represent a suitable microenvironment for proliferation and cohesiveness of endodermic (Takai et al. 2001) and mesenchymal cells (Chernousov et al. 2001; Grotendorst et al. 1981; Ruggiero et al. 1994). On the contrary, an anti-cohesive and anti-proliferative property of this collagen type is evoked when endothelial cells are involved in the process (Ruggiero et al. 1994). In fact, our group demonstrated an inverse and different action of Col V in the extracellular matrix from that observed in lung cancer. We found that rabbits immunized with human Col V, in an attempt to produce the corresponding antibodies, developed increased Col V synthesis by fibroblasts and consequent fibrosis in vessels and lungs (Callado et al. 2007; Teodoro et al. 2004; Velosa et al. 2010).

The current study also disclosed that the increased Col V protein and gene expressions after nasal administration of Col V was associated with increased epithelial and endothelial cell apoptosis caspase-dependent. This finding reinforce the idea that an augment of Col V bindings to epitopes on cell surface, promotes the synthesis of Col V by these cells, superimposed to fibroblasts and myofibroblasts also present tumor and non-tumor stroma (Luparello and Sirchia 2005; Parra et al. 2010). Our results provide information, at the molecular level, showing that an increase amount of Col V may lead to decrease angiogenesis by sufficient caspase-3-induced endothelial apoptosis (Kim et al. 1993; Mattern et al. 1995; Sgonc 1999). As Col V is also a known angiogenic factor in

various cell lines (Montesano et al. 1983; Sage and Vernon 1994) we evaluated whether anti-Col V immunity by nasal administration of Col V may limit the lung tumor angiogenesis via VEGF pathway. We found no difference of VEGF genic expression by tumors in animals which receive nasal administration or not. This finding emphasize that Col V administration reduce tumor growth and angiogenesis more by epithelial/endothelial apoptosis caspase-dependent than VEGF pathway.

One of the limitations of our study is the absence of a dose response curve of the nasally administered Col V in the cancer—Col V mice. In our previous works with Col V in human and experimental pulmonary fibrosis, connective tissue diseases and lung cancer (Bezerra et al. 2006; Garippo et al. 2007; Ogido et al. 2008; Parra et al. 2006, 2010; Souza et al. 2010; Teodoro et al. 2003, 2004; Velosa et al. 2007, 2010) the satisfactory dose to acquire the first response after Col V intranasal administration is 3–30 μg and is in concordance with the experience of other group's (Bobadilla et al. 2008; Braun et al. 2009; Lott et al. 2013; Mizobuchi et al. 2003; Yamada et al. 2009; Yasufuku et al. 2001, 2002; Yoshino et al. 1995). A pilot study was done in the present study (unpublished data) that confirmed Col V dose of 20 μg to acquire lung cancer experiments and that is the reason why we have chosen this concentration. Another question involves how Col V is able to mediate tolerance to an irrelevant nasal peptide, such as ovoalbumin. Previous studies have shown that Col V-induced tolerance to unrelated antigens, such as alloantigens in lung transplantation, is mediated by linked suppression and via induction of regulatory T cells (Lott et al. 2013). The further determination and delineation of the mechanism by which Col V-induced tolerance alters lung tumors represent new studies that will be performed over the next several years.

In conclusions, intranasal administration of Col V inhibited tumor growth, altered extracellular matrix Col V synthesis and induced apoptosis by cleaved caspase-9 in epithelial and endothelial cells. These results suggest that the pathway related to cell death is involved in the administration of murine urethane-induced lung carcinogenesis by intranasal administration of Col V.

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