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Caveolin-1 localization in chicken embryo chorioallantoic membrane treated with diamond and graphite nanoparticles

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Abstract: *Caveolin-1 localization in chicken embryo chorioallantoic membrane treated with diamond and graphite nanoparticles*. Caveolin--1 is a multifunctional protein and major component of caveolae membranes that participates in regulation of signaling pathways, endocytosis and molecular transport. Caveolin-1 takes part in regulation of angiogenesis regulation signaling pathways. Diamond nanoparticle have been shown to inhibit development of blood vessel. Molecular mechanism of diamond nanoparticles anti-angiogenic activity can be related with interactions with cellular membranes. The objective of this experiment is to verify effect of carbon nanoparticles on morphology of highly vascularized chicken embryo chorioallantoic membrane (CAM) and caveolin-1 intracellular localization. In this study two types of carbon nanoparticles were used: diamond nanoparticles (ND) and graphite nanoparticles (NG), which are similar in size (3–5 nm) but different in molecular structure. At day six of chicken embryo embryonic development sterile implant of diameter 10 mm made from Waterman filter paper ware placed on chicken embryo CAM. At day seven of embryonic development implants with CAM were subjected to further analyzes. CAM cross-sections were immuno-localized with anti caveolin-1 antibody and visualized by confocal microscope. Three dimensional analysis of chorion membranes show that ND, but no NG change intracellular distribution of caveolin-1. Furthermore ND decreases density of mesenchymal cells and extracellular matrix collagen fibers.

Key words: caveolin-1, angiogenesis, diamond nanoparticles, graphite nanoparticles, embryo, chicken, confocal microscope.

INTRODUCTION

Caveolin-1 is a multifunctional protein and a major component of caveolae membranes that participates in regulation of signaling pathways, endocytosis and molecular transport. Caveolin-1 is a main protein of caveolae membrane complex. Caveolae are omega-shape invagination in the cell membrane, 60 to 80 nm in size, containing besides caveolins cholesterol and sphingolipids (Stan 2005). Caveolin-1 down-regulate main pro-angiogenic factor VEGF and Akt and Stat3 signaling pathways, that are important in development of new blood vessels (Bauer et al. 2005, Li et al. 2011). Signaling capability of caveolin-1 is based on interaction between it conserved scaffolding domain (amino acids 82–101) and signaling molecules like Gα subunits and other related Src family tyrosine kinases (Li et al. 1996). However, caveolin-1 scaffolding domain also has membrane-binding activity and binding with membrane reduces interaction with signaling molecules (Epand et al. 2005). Caveolae can provide a reservoir of caveolin molecules that are released under specific cellular conditions, for example acute mechanical stresses (Sinha et al. 2011). Cellular poll of caveolin-1, not associated with caveolae can be essential for its signaling capabilities.

Our recent reports show that diamond nanoparticles decease mRNA level of VEGF and other pro-angiogenic factor FGF2 and decrease vesicular network density in glioblastoma tumor (Grodzik et al. 2011). Carbon nanoparticles also decrease induced by VEGF angiogenesis on chicken embryo chorioallantoic membrane (CAM) (Murugesan et al. 2007). Molecular mechanism of diamond nanoparticles anti-angiogenic activity can be related with its interactions with cellular membranes. We hypothesize that carbon nanoparticles effects cellular pool of caveolin-1, that can down-regulate cellular angiogenic signaling pathways. The objective of this experiment is to verify effect of diamond and graphite nanoparticles on morphology of well described, highly vascularized chicken embryo CAM (Hamburger and Hamilton 1951) and caveolin-1 intracellular localization.

METHODS AND MATERIALS

In this study two types of carbon nanoparticles were used: diamond nanoparticles (ND) (explosion synthesized, size: 3–4 nm, specific surface area: ~282 $m^2 \cdot g^{-1}$, purity: > 95%) graphite nanoparticles (NG) (explosion synthesized, size: 3–5 nm, specific surface area: 540–650 $m^2 \cdot g^{-1}$, purity: > 93%). Nanoparticles were obtained from Skyspring Nanomaterials (Houston, USA). Nanoparticles were dispersed in demineralized water with 60 min of sonification.

Fertilized eggs from Ross line 308 hens were obtained from certified hatchery and kept for four days at 12°C. The eggs were cleaned, sterilized with UVC light and divided into three groups (3 × 15 eggs). Embryos were incubated at standard conditions (temperature 37°C, humidity 60%, turned once per hour). At day six of chicken embryo embryonic development sterile implant of diameter 10 mm made from Waterman filter paper were placed on chicken embryo chorioallantoic membrane. Demineralized water (control) or hydrocolloidal nanoparticles of concentration 500 $mg \cdot l^{-1}$ were added on implants (final amount of nanoparticles on implant 0.01 mg). Paper discs were pretreated with 100 $mg \cdot ml^{-1}$ hydrocortisone sodium succinate and air dried under sterile conditions. Chicken embryos were incubated to day seven of embryonic development, when implants with CAM were prefixed with 1.5 ml of 4% paraformaldehyde. After 30 minutes of incubation in 4°C CAM with implants were cut out and fixed in 4°C 4% paraformaldehyde for 60 minutes (total fixation time 90 min). After fixation implants were gently stripped off.

For confocal immuno-fluorescent analysis CAM was frozen in Jung Tissue Freezing Medium (Leica, Wetzlar, Germany) in liquid nitrogen and cut into 5-μm-thick sections using cryostat (CM 1900, Leica, Wetzlar, Germany). The sections were attached to poly-l-lysine coated microscope slides. Tissue was washed with PBS and permeabilized with 0.5% Tween 20 (Sigma, St. Louis, USA) PBS solution for 10 min. The sections were blocked with PBS containing 2% of goat serum and 1% bovine serum albumin (Sigma, St. Louis, USA) for 30 min. Sections were incubated with primary antibody (rabbit anti Caveolin-1 antibody; Sigma, St. Louis, USA) diluted in 2% goat serum (producer recommended dilution) for 12 hrs in 4°C. After

washing sections were incubated with secondary antibody: goat anti-rabbit Atto 488 conjugate (IgG (H+L),F(ab')2 Fragment Atto488; Cell Signaling Technology, Danvers, USA) for 2 hrs, diluted according to producer instructions. Nuclei were stain by incubation with DAPI solution for 15 min (Sigma, St. Louis, USA). After washing the coverslips were mounted on slides with Fluoromount mounting medium (Sigma, St. Louis, USA) and observed on IX 81 FV-1000 confocal microscope (Olympus Corporation, Tokyo, Japan). Image analysis in confocal mode, Nomarski interference contrast, cell counting were performed using FVIO-ASW ver. 1.7c software (Olympus Corporation, Tokyo, Japan). Three-dimensional images were assembled from 30 optical sections.

Data analysis was performed using one-way analysis of variance (ANOVA) with the Bonferroni post hoc test used for multiple comparisons. Values differing at $P < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Analyzed CAM cross-sections show lower density of mesenchymal cells in ND treated group in comparison to control and NG treated group (Table 1). There was not statistically significant reduction of mesenchyme cells in NG treated group. Moreover analysis of Nomarski interference contrast images show that ND treated group have lower density of collagen then control and graphite treated group (Fig. 1). Consequently less developed connective tissue with low density of extracellular matrix in ND treated group can led to anti-angiogenic activities. ND toxicity was demonstrated on in vivo rat model, where ND decreased the number of the phagocytizing neutrophils and increased number of cells with stimulated oxidative burst (Niemiec et al. 2011). Furthermore, ND can led to up-regulation of SOD1 expression responsible for defensive mechanisms against reactive oxygen species in human neoplasmic cells cultured in vitro (Bakowicz-Mitura et al. 2007).

TABLE 1. Number of mesenchyme cells per 1 mm^2 on CAM treated with nanoparticles in comparison to control

Group	Number of mesenchyme cells per 1 mm^2
Control	5.8 [a]
ND	2.9 [b]
NG	4.8 [a]
P-value	0.002
SE-pooled	0.54

Values within rows with different superscripts are significantly different $P < 0.05$.

Confocal microscope images of CAM cross-sections from control, NG and ND treated group were analyzed for expression of caveolin-1. Mesenchyme cells, endothelium of blood vessels, chorion membrane showed expression of cavaeolin-1 in all analyzed groups. Chorion membranes showed the strongest expression of caveolin-1 regardless of group (Fig. 2). Three dimensional analysis of chorion membranes showed differences of caveolin-1 localization between groups. In the control and NG treated groups caveolin-1 was mainly localized on side parts of membrane (Fig. 3). In 80% of analyzed images of ND treated chorion membranes caveolin-1 was evenly distributed across cells. Intracellular caveolin-1 can down-regulate important

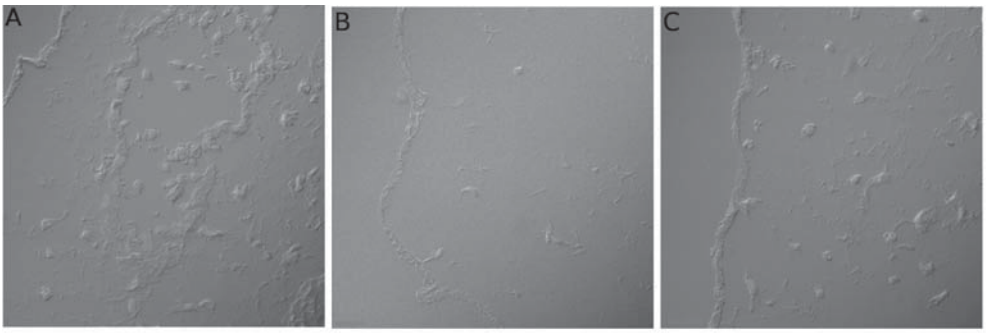

FIGURE 1. Nomarski interference contrast image of CAM tissue extracellular matrix. Nuclei are stained with DAPI A – Control, B – ND, C – NG. Scale bar 50 μm

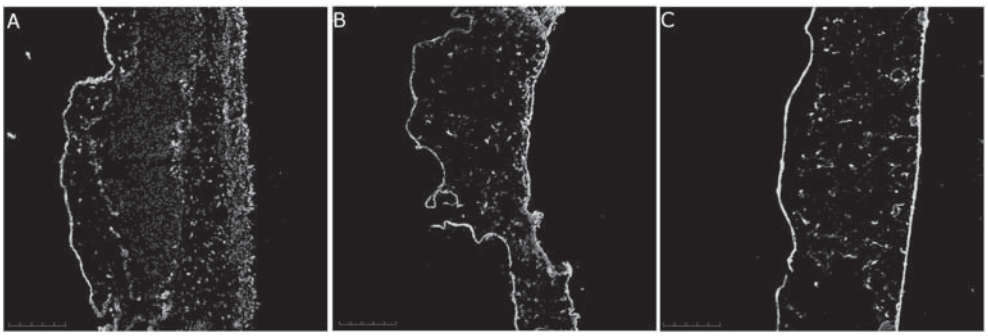

FIGURE 2. Chicken embryo CAM tissue with immune-localized caveolin-1 and stained nuclei with DAPI. A – Control, B – ND, C – NG. Scale bar 200 μm

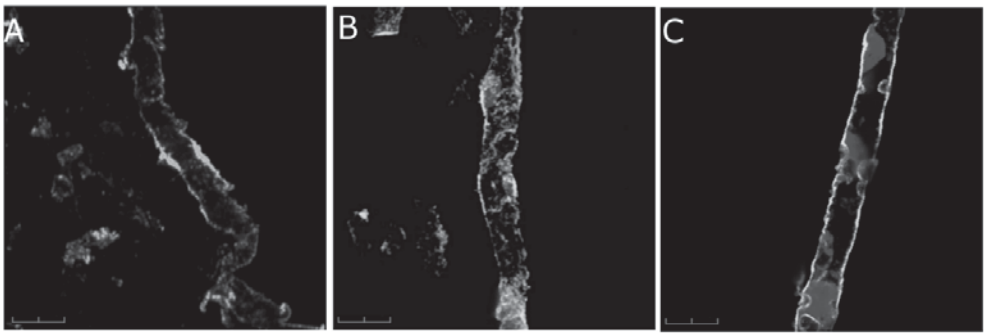

FIGURE 3. CAM chorion with immune-localized caveolin-1 and stained nuclei with DAPI. A – Control, B – ND, C – NG. Scale bar 10 μm

in angiogenesis Akt and Stat3 signaling pathways (Li et al. 2011). Caveolin-1 change of localization can by associated with interactions with proteins localized in caveolar membranes, such as receptor tyrosine kinases (receptor of VEGF – VEGFR2), Serine/Threonine, Src family kinases (Parton and Simons 2007) and caveolae-mediated endocytosis of carbon nanoparticles (Zhang et al. 2009). Moreover, ND could act by direct acute mechanism, releasing caveolin molecules from caveolae as shown by Sinha et al. (2011). Very similar in size, but different in molecular structure NG did not show distinct effects, which indicate that interaction of ND is specific for this nanoparticle. Obtained results give new insights in carbon nanoparticles interactions with biological systems. ND change structure of CAM and show inhibitory effect on connective tissue. Changes in caveolin-1 localization in ND treated group indicate potential angiogenic regulation mechanism.

CONCLUSIONS

Immuno-localization of the control, ND and NG treated chicken embryo CAM showed expression of caveolin-1. ND changed intracellular distribution of caveolin-1 in chorion membranes. Furthermore, ND decreased density of mesenchymal cells and extracellular matrix collagen fibers. The results indicate that caveolin-1 can be associated with ND anti-angiogenic properties.

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Streszczenie: *Lokalizacja kaweoliny-1 w błonie kosmówkowo-omoczniowej zarodka kury traktowanej nanocząstkami diamentu oraz grafitu*. Kaweolina-1 jest wielofunkcyjnym białkiem, będącym składnikiem błonowych kaweoli, biorącym udział w regulacji szlaków sygnałowych, endocytozy oraz transportu wewnątrzkomórkowego. Kaweolina-1 uczestniczy między innymi w regulacji szlaków sygnalnych związanych z angiogenezą. Wcześniejsze badania wykazały, że nanocząstki diamentu mają zdolność hamowania rozwoju naczyń krwionośnych. Molekularny mechanizm właściwości anty-angiogennych związany jest prawdopodobnie z interakcją nanocząstek z błonami komórkowymi. Celem tego doświadczenia jest zbadanie efektów działania nanocząstek na morfologię błony kosmówkowo-omoczniowej (CAM), oraz wewnątrzkomórkową lokalizację kaweoliny-1. W badaniach wykorzystane zostały dwa rodzaje węglowych nanocząstek: nanocząstki diamentu (ND) oraz nanocząstki grafitu, charakteryzujące się podobną wielkością (3–5 nm), ale posiadające inną budowę molekularną. Szóstego dnia inkubacji zarodka kury sterylny implant o średnicy 10 mm, wykonany z papieru filtracyjnego Waterman został położony na CAM. Siódmego dnia rozwoju zarodkowego implanty razem z CAM zostały pobrane do dalszych analiz. Przekroje poprzeczne CAM zostały wyznaczone przeciwciałami anty kaweolina-1 i obserwowane pod mikroskopem konfokalnym. Analiza trójwymiarowych zdjęć błony kosmórkowej wykazała, że ND, ale nie NG zmienia wewnątrzkomórkową lokalizację kaweoliny-1. Ponadto ND zmniejsza gęstość komórek mezenchymalnych oraz ilość macierzy zewnątrzkomórkowej złożonej z włókien kolagenowych.

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