Journal of Glycobiology
ISSN: 2168-958X

Borba et al., J Glycobiol 2017, 6:3
DOI: 10.4172/2168-958X.1000129

Research Article Open Access

Pro-angiogenic Activity Assay of Chondroitin Sulfate and Glucosamine Sulfate on Vascular Network of Mouse and of Chick Embryo Chorioallantoic Membrane

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Received date: October 04, 2017; **Accepted date:** October 26, 2017; **Published date:** October 31, 2017

Abstract

Objective: Target of this study was to test the capacity of chondroitin sulfate (CS) and glucosamine sulfate (GS) to induce *in vivo* angiogenesis.

Methods: The proangiogenic activity of these compounds was analyzed through the assays in chorioallantoic membrane (CAM) of chick embryo and dorsal skin vascularization in mice, but before was realized a cell viability assay with human umbilical veins endothelial cells (HUVEC).

Results: In the viability assay, concentrations tested between 30 and 3000 µg/ml showed a reduction of viable HUVEC number. In the CAM assay, CS and GS in an amount 2.0 mg/implant increased the vessels number as compared to control (phosphate buffered saline-PBS). In the assay of the dorsal skin vascularization of adult Swiss mice, the groups treated with CS (2 mg/implant; Gelfoam plug) exhibited an increase in the vessels number into plugs (0.52 ± 0.08 g/dl; measured as plug-hemoglobin content), a similar effect to that promoted by fibroblast growth factor-2 (FGF-2; 50 ng/implant) (0.53 ± 0.1 g/dl). However the group treated with GS did not exhibit significant effect on mice skin vascularization.

Conclusion: CS was capable to promote angiogenesis on CAM and dorsal skin vascularization, but GS only had pro-angiogenic activity in CAM vascular network.

Keywords: Chondroitin sulfate; Glucosamine sulfate; Angiogenesis; Chorioallantoic membrane; Matrigel plug

Abbreviations: ANOVA: Analysis of Variance; CAM: Chorioallantoic Membrane; CS: Chondroitin Sulfate; CSPG: Chondroitin Sulfate Proteoglycan; D_{BC}: Box-Counting Dimension; DMSO: Dimethyl Sulfoxide; FBS: Fetal Bovine Serum; FGF-2: Fibroblast Growth Factor-2; GAG: Glycosaminoglycan; GS: Glucosamine Sulfate; HS: Heparin Sulfate; HSPGs: Heparin Suphate Proteoglycans; HUVEC: Human Umbilical Veins Endothelial Cells; ICAM-1: Intercellular Adhesion Molecule-1; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; NG2: Neuron-glial antigen 2; NRP1: Neuropilin-1; PG: Proteoglycan; PBS: Phosphate Buffered Saline; PDGFB: Platelet-Derived Growth Factor Subunit B; RPMI: Roswell Park Memorial Institute; SEM: Standard Error of the Median; VEGF: Vascular Endothelial Growth Factor; VEGFR: Vascular Endothelial Growth Factor Receptor; YSM: Yolk Sac Membrane; TGF-β: Transforming Growth Factor β

Introduction

Angiogenesis is the blood vessels sprouting from the pre-existing vessels, leading vascular network remodeling [1,2]. This remodeling is characterized by luminal diameter expansion of newly formed vessels in response to increased blood flow [3,4]. The formation of new blood vessels involves steps such as proteolytic degradation of extracellular matrix, migration by chemotaxis, adhesion, proliferation and differentiation of endothelial cells; finally emerging a new tubular structure to the bloodstream [5-7]. Addition of the endothelial cells, the mural cells (muscle cells and pericytes) participate in the vessels morphogenesis guided by growth factors interaction with their receptors; furthermore also there is a contribution of other molecules to blood vessels formation [8-10].

Angiogenesis is stimulated by growth factors, including particularly vascular endothelial growth factor (VEGF) and FGF-2 [11-13]. These growth factors promote several angiogenesis steps, including endothelial cells interaction with extracellular matrix and degradation of this matrix by own endothelial cells [14,15]. This interaction between growth factors with their respective receptors is necessary to promote the angiogenesis. Some polysaccharides can be involved in this interaction, they are known as glycosaminoglycans (GAGs) [16].

GAGs are unbranched heteropolysaccharides composed of repeating disaccharide units that consist of either sulfated or non-sulfated monosaccharides [17,18]. Disaccharide repeating units are composed uronic acids (D-glucoronic acid or L-iduronic acid) and

amino sugar (D-galactosamine or D-glucosamine) [19]. GAGs can differ according to the sulfation, as well as the presence of amino sugars and uronic acids [18]. Non-sulfated GAGs include hyaluronic acid, whereas sulfated GAGs can possess galactosamine in their chains (CS and dermatan sulfate), glucosamine (heparin and heparin sulfate-HS) [18] or to contain galactosamine without uronic acids (keratan sulfate) [18].

GAGs as HS and CS may be attached to a core protein constituting a proteoglycan (PG) that is able to regulate the growth factors activities [16,17,20,21]. HS with its PGs (HSPGs) regulate angiogenesis through capability to bind to growth factors (VEGF and FGF-2) and also to their receptors [16]. Binding of VEGF165 to HS is mediated by amino acid C-terminal heparin binding domain, meanwhile the connection of HS with VEGF receptor 2 (VEGFR2) probably is realized through a ten amino acid sequence between Ig-like domains 6 and 7 of this receptor [22]. The heparin binding domain of FGF-2 is discontinuous and located in the N-terminal and in the C-terminal part, while the heparin binding site to FGF receptor 1 (FGFR1) is contained in the Ig-like module II of this receptor [22].

PGs are localized on cellular surface, basement membrane or extracellular matrix [17]. Syndecans (syndecan-1 to 4) and betaglycan are examples of PG (compound of HS and CS) present on cellular membrane [23-25]. Neuropilin-1 (NRP1) also is a membrane PG of endothelial cells, formed for CS or HS [25], NRP1 is VEGFR2 co-receptor [26,27] and is essential to embryonic angiogenesis and vascular development [28]. NRP1 can interact with heparin-biding isoforms VEGF-A, VEGF-B and VEGF-E [29].

Our aim was to test the capacity of CS and GS to induce angiogenesis, utilizing CAM of chick embryos (embryonic angiogenesis) and adult mice skin (advanced angiogenesis).

Materials and Method

Materials

The materials utilized in assay were: CS and GS, both with purity above 90% (Phytomare Company, Governador Celso Ramos, SC, Brazil); dimethyl sulfoxide (DMSO), FGF-2 (F0291), methylcellulose, were purchased from Sigma-Aldrich (St. Louis, MO, USA); MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Invitrogen (Grand Island, N.Y., USA); FBS, L-glutamine, penicillin/streptomycin, RPMI (Roswell Park Memorial Institute) 1640 medium were purchased from Gibco (Auckland, New Zealand); Gelfoam (Pharmacia and UpJohn, Kalamazoo, Mi, USA).

Cell viability assay

Toxicity test was performed on HUVEC. The HUVEC were seeded (5.0×10^6 cells/well) in RPMI-1640 medium with 2% FBS, 1% antibiotic (penicillin 1000 UI/ml+streptomycin 250 mg/ml) and 1% L-glutamine 200 mM. The cells suspension was distributed in culture plate with 96-well plates. The plates were incubated at 37°C in CO_2 incubator. After 24 h of incubation, the cells were treated with increasing concentrations of CS and GS (1-3.000 µg/ml). After 3 days, the medium was removed and wells were washed several times with 100 µl of PBS. Next, the evaluation of viability was carried out according to Carmichael et al. [30] and Dias et al. [31], by colorimetric essay of MTT. The RPMI-1640 medium was added to MTT solution (5 mg/ml in PBS) at a ratio of MTT solution to medium of 1:10. The plates were incubated in a humidified atmosphere containing 5% CO_2 at 37°C for 4 h, the medium was aspired and 200 ml DMSO was added to each well to dissolve the formazan crystals. The plates were agitated on a plate shaker for 5 min and the absorbance at 540 nm was determined using a spectra rainbow microplate reader (Tecan, Männedorf, Switzerland). The results are expressed as percent of control (incubation of HUVEC in RPMI-1640 medium alone). All experiments were performed in triplicate.

Animals

All animal studies were carried out in accordance with the procedures outlined in protocol number PP00586/2011/CEUA/UFSC, approved by the Local Committee for Care and Ethical Use of Animals in Research (CEUA/UFSC, Florianopolis, SC, Brazil).

CAM assay

Pathogen-free fertilized chicken eggs (Ross strain, n=6 per experimental group, n=48 in total) were supplied by poultry producers (Tyson S.A., São José, SC, Brazil).

The capacity of CS and GS to stimulate *in vivo* angiogenesis was determined by the CAM of chick embryo, performed according to the method presented by Dias et al. [31]. The eggs were incubated at 37.5°C and 70% humidity. After 48 h of incubation, a window (10 mm diameter) was opened in the eggshell at a position adjacent to the embryo. The treatment in ovo was performed by implanting disk-shaped methylcellulose supports (7.5 µl volume, 3 mm diameter; one disk per embryo) containing only each of the compounds: PBS, CS, GS and FGF-2. The methylcellulose disks were implanted on the outer one-third surface of the CAM of 6 day old embryo (E6), where blood capillaries were still growing. After implanting the disks, the windows were closed with black binding cellophane tape. The eggs stayed in incubation until E8. The concentration of CS and GS administered on CAM vascular network ranged from 0.02 to 2.00 mg/disk. Methylcellulose disks containing FGF-2 (50 ng/disk) and disks containing PBS were used as positive and negative controls, respectively. For each egg, images were captured with a Motic 1000 1.3 MP camera (Motic, Causeway Bay, Hong Kong, China) coupled to a stereomicroscope (20x magnification). The vessels in the region around the limit of disk were quantified by calculating the fractal dimension (Figure 1).

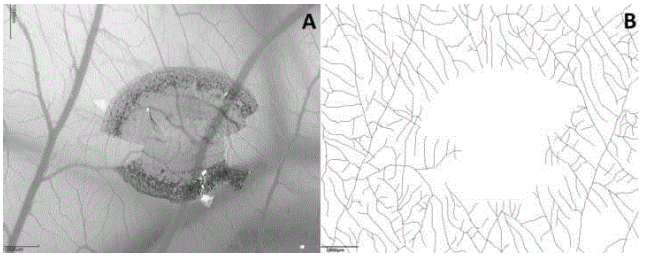

Figure 1: Image skeletonization of CAM vascular network. Image of CAM vascular network with methylcellulose disk (A) and its respective skeletonized image (B).

Image skeletonization of vascular network

The digital images (1240 × 1024 pixels) of vascular network were manually skeletonized using Microsoft Paint to separate the blood

vessels from the rest of the images. Each vessel was traced by a line that was 1 pixel thick, then, the images were binarized, resulting white vessels on black background [32,33].

Fractal analysis

The method used to calculate the fractal dimension of CAM vascular network was box counting dimension (D_{BC}). The obtainment of D_{BC} values was calculated by Benoit 1.3 Fractal Analysis System software (Trusoft, St. Petersburg, FL, USA). The D_{BC} was obtained by covering the skeletonized image of CAM vessels with N (r) boxes containing at least one point of the image and then repeating with boxes of different sizes. The value of the slope of a plot of a double log of N (r) in function on the sides of boxes r [33] is the D_{BC} and can be calculated through the following equation:

$$D_{BC} = -\lim_{\varepsilon \to 0}\left[\frac{\log N(r+\varepsilon) - \log N(r)}{\log(r+\varepsilon) - \log(r)}\right]$$

where ε is the small variation in the box size.

Gelfoam plug assay

This *in vivo* assay was based in the method described by Dias et al. [Dias]. Male Swiss mice (Mus musculus), pathogen-free, were acquired from the Central Biotery of Federal University of Santa Catarina (Florianopolis, Brazil). The male Swiss mice of 8 weeks old with an average body weight of 27 ± 2 g were housed in a light-controlled room (lit from 7:00 A.M. to 7:00 P.M.) with temperature of 24 ± 1°C and fed on sterilized animal feed and water ad libitum.

The groups were treated with CS, GS (2 mg/animal) and FGF-2 (50 ng/animal). PBS (vehicle) and FGF-2 were used, respectively, as negative and positive controls. Each compound was adsorbed (50 µl) in a sterile compressed sponge (Gelfoam plug; 6 mm diameter × 3 mm). Gelfoam plug was implanted subcutaneously into the rear right flank of a mouse (n=6). The mice were killed 2 weeks later by CO_2 inhalation and the skin was carefully pulled away to expose the intact Gelfoam plug. The amount of hemoglobin inside the Gelfoam plug was measured using Drabkin reagent as a quantifiable index of blood vessel formation [34]. The hemoglobin concentration is expressed as milligrams per deciliter. The calculation is based on a hemoglobin standard measured simultaneously using the following equation described by Lee et al. [35]: sample absorbance/standard absorbance × 10.

Statistical analysis

Experimental data of each assay were evaluated by ANOVA and Tukey´s Post Hoc test. Effect were considered to be statistically significant at $p<0.05$. Experimental data were summarized and expressed as mean ± SEM.

Results

Effects of CS and GS on HUVEC viability

We evaluated the effect of increased concentrations of CS and GS (1-3.000 µg/ml) on HUVEC lineage cells viability by the MTT toxicity assay. After 48 h treatment, the MTT assay showed that the HUVEC viability was not statistically significant to the treatments between 1-10 µg/ml to both CS and GS (Figure 2). When the treatment was increased from 30 to 3000 µg/ml, was observed that there was reduction of HUVEC viability. In concentration of 30 µg/ml, the CS and GS reduced the amount of viable cells to 65.5 ± 1.25% and 60.3 ± 2.41%, respectively, when compared to control group (100%), to $p<0.05$. To highest concentration tested (3000 ug/ml), CS reduced the HUVEC viability to 53 ± 2.87%, while GS reduced the cellular viability to 36.5 ± 1.8%. Figure 2 shows the effect for administration of CS and GS in all tested concentrations.

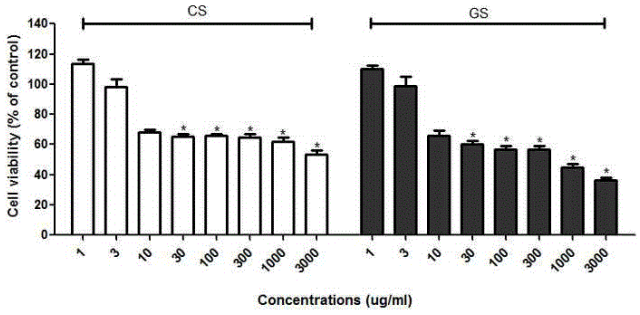

Figure 2: Effects of CS and GS (1-3000 µg/ml) on HUVEC line cells viability (5.0×10^6 cells/well). After 48 h of exposition to the test compounds, the percentage of viable cells was determined by incubation (4 h) with MTT. The experiments were done in triplicate, and the results are expressed as percentage of the control group (medium: RPMI-1640). Each column with respective vertical bar represents mean ± SEM, the columns with asterisk denote statistically significant differences with $p<0.05$, in relation to the control group (ANOVA followed by Tukey test).

CAM assay

CAM assay was used to determine whether CS and GS displayed pro-angiogenenic activity. We evaluated the vascularization around the methylcellulose disk in chick embryo CAM (implanted at E6). Figure 3 show the results of fractal dimensions D_{BC} for the groups treated with CS and GS, respectively, at E8.

Figure 3A and 3B display a significant increase ($p<0.05$) in the density of blood vessels to the highest tested concentration (2 mg/disk) of CS (D_{BC}=1.30 ± 0.03) and GS (D_{BC}=1.28 ± 0.04) when compared to the negative control group (D_{BC}=1.19 ± 0.03). In addition, this concentration of CS and GS had a similar effect to the FGF-2 (D_{BC}=1.34 ± 0.03) in promoting neovascularization. However, significant difference ($p<0.05$) was no observed for the fractal dimensions at concentrations of CS and GS below 2 mg/disk, compared to the negative control group (PBS).

Gelfoam plug assay

We conducted an assay in dorsal subcutaneous vascularization of mice with 8 weeks in order to test whether the pro-angiogenic effects of CS and GS, previously observed in embryonic stage, would also be observed in adult individuals (advanced angiogenesis). In this experiment, we evaluated the formation of blood vessels based on the amount of hemoglobin in the Gelfoam plug implanted under the animal skin.

At the 15th experiment day, negative control group (PBS-treated Gelfoam plugs) clearly showed new blood vessels, with hemoglobin content in the plug of 0.20 ± 0.07 mg/dl. However, Gelfoam plugs

containing FGF-2 (positive control) displayed a higher hemoglobin content (0.53 ± 0.1 mg/dl) than negative control ($p<0.01$, Figure 4). Group treated with CS (2 mg/implant) displayed a higher vessels growth (hemoglobin content of 0.52 ± 0.08 mg/dl) than negative control group ($p<0.01$). While GS did not induce a significant vessels growth (0.37 ± 0.09 mg/dl) compared to the negative control group.

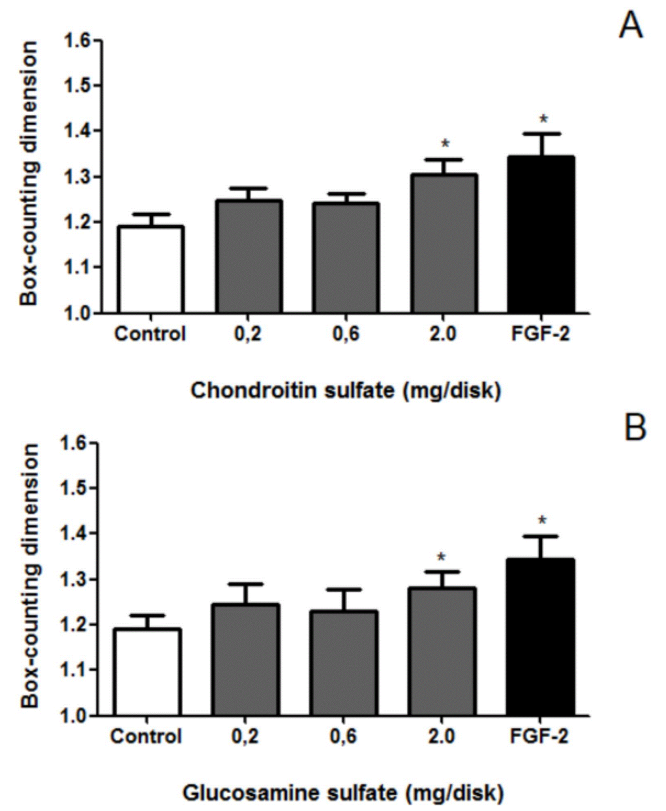

Figure 3: Stimulatory effect to concentrations of 0, 2-2 mg/ml of both chondroitin sulfate (A) and glucosamine sulfate (B) on vascularization of the 8 day YSM. Results are quantified by box-counting fractal dimension (D_{BC}). The controls were performed with vehicle (PBS, negative control) and FGF-2 (50 ng/disk, positive control). Each column with respective vertical bar represents the mean ± SEM of six eggs. $^{*}p<0.05$, versus negative control (ANOVA and Tukey as a post hoc test).

Discussion

Glucosamine is an aminomonosaccharide which constitutes some GAGs which form the matrix of all connective tissues including articular cartilage, while CS is a GAG linked sulfate group and also composes the articular cartilage [36]. Some studies have reported pro [21,32,37] and anti-angiogenic effects [38-40] of CS, well as pro-angiogenic effect of GS [32,41,42]. Here, we show that concentrations of CS and GS in the range of 30-3000 µg/ml promoted toxicity to HUVEC (5.0×10^6 cells). GS had a higher toxic effect on cells than CS.

A concentration of 2000 µg/disk of CS and GS was capable to stimulate neovascularization in the CAM of chick embryo, while lower concentrations of these molecules did not have significant effect (Figure 3). In Gelfoam plug implanted under mice skin, CS showed a significant effect on the amount of erythrocytes in relation to control group. Nevertheless, the GS did not show significant effect to Gelfoam plug assay. According to *in vivo* assays, there is a threshold concentration of CS and GS to perform angiogenic activity, well as a high concentration can result in toxic effects to cells as shown by *in vitro* assay.

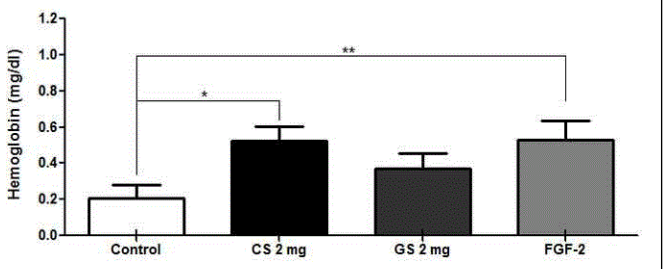

Figure 4: Effects of CS and GS (2 mg/animal), as well as combined with FGF-2 (50 ng/animal) in subcutaneously implanted Gelfoam plugs in adult mice, based on hemoglobin content in the plug. The test implants were adsorbed from 50 µl of FGF-2 (50 ng), with or without CS or GS. The negative and positive controls consisted of, respectively, 50 µl of PBS (vehicle) and FGF-2 (50 ng). Each column with respective vertical bar represents the mean ± SEM for at least 5 animals, and the asterisks denote statistically significant differences for $^{**}P<0.01$.

In relation to the angiogenic activity, CS provoked a higher effect on vascular density in relation to the GS, as shown in the CAM and Gelfoam plug assay. We have observed in our previous work that CS and GS were capable to stimulate vasculogenesis and angiogenesis in the yolk sac membrane (YSM) of chick embryo through fractal analysis [32]. However 1 µg/ml of GS produced a similar increase in the vascular network obtained by a dose of 100 µg/ml of CS [32], thus contradicting the results shown in the current paper in which the angiogenic activity of CS was higher, according to the fractal dimension and amount of hemoglobin obtained in Gelfoam plug assay. GS had a more effective action on vasculogenesis and early angiogenesis (YSM assay and CAM) than later angiogenesis (Gelfoam plug assay). The availability of GS at the emergence and initial growing of vessels seems to contribute more to expansion of the vascular network than at later angiogenesis. Since GS can participate in the HSPGs synthesis by vascular endothelium, thus contributing to vessels development [22].

On the other hand, CS presented a higher action on mature angiogenesis. The CS is a heteropolysaccharide which can bind to pro-angiogenic factors such as VEGF-A, TGF-β (Transforming growth factor β), PDGFB (Platelet-derived growth factor subunit B) [21]. The CS can act on signaling of TGF-β, this growth factor is able to maintain the endothelial cells quiescence, induces vessels maturation and influences expression and angiogenic factors activities like VEGF [21,43]. CS is capable to regulate growth factors-mediated cell migration implicating in tumor angiogenesis [24]. The study realized by Le Jan et al. [21] also indicates the involvement of CS in angiogenesis sprouting. CSPGs are able to modulate several steps of angiogenesis [21]. There is a CSPG known as neuron-glial antigen 2 (NG2) that is expressed on the surface of pericytes during vasculogenesis and angiogenesis [44]. This CSPG is considerable element in promoting endothelial cells migration and morphogenesis

in the early stages of neovascularization [45]. Also Tapon-Bretaudière et al. [37] reported an increase of vascular tubes formation by endothelial cells in the presence of FGF-2 in HUVECs treated with the fucosylated CS obtained from sea cucumber, which has similar chemical structure to mammalian CS.

Conclusion

In our study, concentrations between 30 and 3000 μg/ml were enough to decrease the HUVEC viability. Fractal analysis of vascular network of chick embryo chorioallantoic membrane revealed the pro-angiogenic effect of chondroitin and glucosamine sulfates (2 mg/disk). Also the evaluation of hemoglobin content in the Gelfoam plug implanted under the mouse skin permitted to identify angiogenic capacity of chondroitin sulfate (2 mg/disk). Nevertheless the Gelfoam plug assay showed that glucosamine sulfate did not have significant angiogenic effect.

Acknowledgement

We thank Dr. Maria Beatriz da Rocha Veleirinho (Plant Morphogenesis and Biochemistry Laboratory/CCA,-UFSC) by the valuable collaboration in the development at this work. We also thank Giovanni Loss and Lisiê Silva by their technical assistance. We are grateful to the Research support center of UFRPE (CENAPESQ) for technical support.

This work was funded by Brazilian support agencies: Coordination for the Improvement of Higher Education Personnel (CAPES), Foundation for Science and Technology Support in Pernambuco (FACEPE), National Council for Scientific and Technological Development (CNPq).

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