Original Article
Content change and clinical significance of oxidation damage products 8-OHdG, 4-HNE and NTY in hemangioma tissue

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Abstract: Purpose: To discuss content change and clinical significance of oxidation damage products 8-hydroxy-2-deoxyguanosine (8-OHdG), 4-hydroxynonenal (4-HNE) and nitrotyrosine (NTY). Methods: This study collected 28 cases of surgical resection and biopsy tissue of hemangioma and 5 cases of normal skin tissue adjacent to cancer from the Department of Pathology of Renmin Hospital of Wuhan University from 2009 to 2014. The expression of 8-OHdG, 4-HNE and NTY in each group was detected and analyzed by immunohistochemistry. Immunofluorescence histochemical technique with a new labeling reagents-quantum dot (QDs) was used to detect the relationship between the expression of 8-OHdG, 4-HNE, NTY in human hemangioma tissue and the occurrence and degradation of hemangioma. The average absorbance and positive area percentage of the immune response in each group were analyzed by One-Way ANOVA and SNK (q) test by using SPSS 13.0. Results: The oxidative damage products 8-OHdG, 4-HNE and NTY in proliferative hemangiomas showed high expression, while showed low expression in normal skin tissue and involution hemangiomas. The statistical analysis showed that the expression of 8-OHdG, 4-HNE and NTY in proliferative hemangiomas were significantly higher than that of the involutional stage of hemangioma and in normal skin tissues (P<0.05). Conclusion: The high expression of oxidative damage products 8-OHdG, 4-HNE and NTY in proliferative hemangiomas makes the vascular endothelial cells hyperplasia, so as to impel the hemangioma formation, which plays an important role in the occurrence and development of hemangioma.

Keywords: Hemangioma, 8-OHdG, 4-HNE, NTY, immunohistochemistry, quantum dots

Introduction

Cutaneous hemangioma is one of the most common benign tumors. Most hemangioma will go through three stages, which is the period of proliferation, degradation and degradation completion period [1, 2]. Although the skin hemangioma is a benign tumor, but the pathological changes is mainly due to the abnormal proliferation of vascular endothelial cells. Parts of the skin hemangioma grow rapidly and can cause defects in appearance and local deformity, which often brings great suffer to patients and their family. There is no clear statement of the pathogenesis of angioma, so for the clinical treatment of skin hemangioma is the urgent need to solve the key problem.

Reactive oxygen species (ROC) is the most important free radicals in the body, which can lead to large biological molecules, such as DNA, lipid and protein, oxidative damaged and generated 8-hydroxy-2-deoxyguanosine (8-OHdG), 4-hydroxynonenal (4-HNE) and nitrotyrosine (NTY), resulting in change of cell function, ultimately leads to damage of tissues and organs function. Therefore, the content of 8-OHdG, 4-HNE and NTY can represent the severity of oxidative damage in cells, which is a recognized oxidative damage marker [3, 4].

This study selected 8-OHdG, 4-HNE and NTY as markers of oxidative damage by free radicals, to observe the expression changes in different stages of hemangioma and to further explore the pathological mechanism of the development and degradation of hemangioma, which can provide experimental basis for clinical treatment of hemangioma.
Hemangioma etiology

Materials and methods

Samples and agents

A total of 28 samples of surgical resection and biopsy tissues of hemangioma were all taken from the Department of Pathology of Renmin Hospital of Wuhan University from 2009 to 2014 (12 males and 16 females, range: 2 months-20 years). Another 5 cases of normal skin tissues were taken as control. This hemangioma where parts of the scalp, eyelids, forehead, ears, neck, arms, back, legs, hands and foot skin. The patients were without any adjuvant therapy.

Ready-to-use mouse-anti-human PCNA monoclonal antibody, Super sensitive ready-to-use immunohistochemical S-P kit, DAB color test box and poly lysine were from ZSGB-BiO, Beijing, China. Mouse-anti-human 8-OHdG monoclonal antibody was from Trevigen (4354-MC-050). Sheep-anti-human 4-HNE polyclonal antibody was from Santa Cruz (SC-130083). Rabbit-anti-human NTY polyclonal antibody was from MILLIPORE (Cat. #06-284). QDs-SA (quantum dot labeled streptavidin complexes) detection kit was from Wuhan Jiayuan Quantum Dots CO. Ltd, China.

Method

Routine HE staining was performed on all specimens and the proliferating cell nuclear antigen (PCNA) was detected by immunohistochemical SP method. All specimens were divided into two groups according to the Mulliken classification standard combined with the expression of PCNA. The specimens were staged according to the characteristics of the organizational structure. There are more than 20% tissues with fat infiltration or fibrosis or vascular occlusion in evolutorial stage of hemangioma, while proliferating stage hemangioma were without the structural changes above. The results showed that 10 cases were in proliferative stage, 18 cases were in evolutorial stage. Another 5 cases of 5 cm peripheral normal skin tissues from cancer tissue were taken as control.

Routine dehydration, transparent, paraffin embedded, sliced and HE staining was performed on all specimens.
Hemangioma etiology

Figure 2. Expression of PCNA in the hemangioma tissue in proliferative and involuting stage (SP, ×200). A. In proliferative stage, the brown-yellow particles were diffuse distribution in the nucleus, the endothelial cell nucleus of hemangioma was hypertrophy and the expression of PCNA was strong; B. In involuting stage, the endothelial cell nucleus was flat, and there was a small amount of brown-yellow particles in a small number of nuclei, and the expression of PCNA was weak.

Figure 3. Expression of 8-OHdG in proliferative and involuting stage hemangioma and normal skin tissue (SP, ×200). A. A large number of brown-yellow particles were found in the nucleus of the endothelial cells and the expression of 8-OHdG was positive in the proliferative stage. B. A small amount of brown-yellow particles were found in the nucleus of the endothelial cells and the expression of 8-OHdG was negative in the involuting stage. C. There is no brown-yellow particles in the nucleus of vascular endothelial cells of normal skin tissues and the 8-OHdG expression was negative.

Immunohistochemical SP method

(1) 4 μm tissue sections were dewaxed routinely into the water; (2) Antigen fixation (the citric acid buffer solution microwave antigen fix method; (3) Dropping 3% H2O2 and the tissues were incubated in 37° wet box for 10 min to inhibit endogenous peroxidase activity; (4) Normal sheep serum were incubated in 37° wet box for 10 min to reduce non-specific background; (5) Dropping primary antibody respectively (PCNA: 1:100; factor VIII: 1:150; 8-OHdG: 1:200; 4-HNE: 1:200; NTY: 1:200); (6) Dropping the streptavidin peroxidase complex and incubated in 37° wet box; (7) Dropping fresh DAB developer to color; (8) Hematoxylin staining; (9) Gradient alcohol dehydration, xylene transparent, neutral balata and microscope; (10) Replace primary antibody with PBS as negative control group, the purchased positive films were as positive control group of 8-OHdG, 4-HNE and NTY group, normal human skin was as the positive control group of PCNA.
Hemangioma etiology

QDs-SA detection

4 μm thick section of hemangioma tissues was dewaxing, hydration, microwave antigen repair and TBS washing. The blocking buffer was incubated in 37°C wet box for 30 min, then dropping 8-OHdG, 4-HNE and NTY antibody, incubated in 37°C for 2 h, TBS-T rinse 3 min for 3 times. The blocking buffer was incubated in 37°C wet box for 10 min, then dropping biotinylated goat-anti-mouse IgG and incubated in 37°C wet box for 30 min, TBS-T rinse 3 min for 3 times. The blocking buffer was incubated in 37°C wet box for 10 min, then dropping QDs-SA diluted with blocking buffer (1:100) and incubated in 37°C wet box for 30-60 min, TBS-T rinse 3 min for 3 times, then dropping 50% glycerol, mount. Fluorescence microscopy was used to excite Qd602 at different wavelengths, and the cells were positive with the orange-red fluorescent particles. Replace primary antibody with TBS buffer as negative control. The whole experiment process was not required to avoid light.

Judgment of the immunohistochemistry and quantum dot staining results

Brown-yellow particles appeared in the nucleus as positive reaction for PCNA, 8-OHdG, 4-HNE and NTY. In negative control group, the nucleus was stained blue, and there was no brown-yellow reaction in the nucleus and cytoplasm. 8-OHdG, 4-HNE and NTY positive reaction sites were expressed with red fluorescence in quantum dot staining.

The expression of 8-OHdG, 4-HNE and NTY were quantitative analyzed by Nuance Fx multi-spectral imaging system from CRI Company, the USA. Each slice was randomly selected 5 complete and non-overlapping high-power field (×400). The average light density, positive reaction area and the total area of all cells in each field were measured, and the positive area rate was calculated. The average light absorbance and the positive area rate of 5 fields of each case were as the measured value for the case (the positive area ratio = total area of the positive reaction in the unit area/total area of the cells in the unit area ×100%).

Statistical analysis

All the data are shown as X ± s. The average light absorbance and positive area ratio of positive reaction particles in each group were analyzed by one-way ANOVA and SNK-q test, the level is 0.05 as a standard test. P<0.05 was considered significant. Statistical analysis was performed with SPSS 13.0 software.

Results

HE staining

The capillary in normal skin tissue is surrounded by 1 to 2 endothelial cells. The wall of the capillary is relatively thin and the endothelial cell is thin, only the nucleus is slightly thicker, and the endothelial cell nucleus id flat (Figure 1A). There are a large number of active endothelial cells in the hemangioma in proliferative stage, which is a cord like or lump. There are irregular endothelial gap and complete vascular cavity in the hemangioma in proliferative stage, and the endothelial cell nuclear is hypertrophy and light staining (Figure 1B). The endothelial cells were significantly reduced in hemangioma in involuting stage and the vascular cavity increases, vascular fibrosis, fatty infiltration and lumen occlusion, endothelial cell nuclear is flat (Figure 1C).

Detection of PCNA by immunohistochemistry

SP method

With brown-yellow particles as the positive judgment standard, the brown-yellow particles were diffuse distribution in the nucleus of the proliferative stage, the endothelial cell nucleus of hemangioma was hypertrophy and the expression of PCNA was strong (Figure 2A). In involuting stage, the endothelial cell nucleus was flat, and there was a small amount of brown-yellow particles in a small number of
nuclei, and the expression of PCNA was weak (Figure 2B).

Combining with the results of HE staining and immunohistochemical SP method detecting PCNA, in 28 cases, there were 10 cases of hemangioma in proliferative stage and 18 in involuting stage.

The expression of 8-OHdG, 4-HNE and NTY detected by immunohistochemistry SP method

The expression of 8-OHdG: A large number of brown-yellow particles were found in the nucleus of the endothelial cells and the expression of 8-OHdG was positive in the proliferative stage (Figure 3A). A small amount of brown-yellow particles were found in the nucleus of the endothelial cells and the expression of 8-OHdG was negative in the involuting stage (Figure 3B). There is no brown-yellow particles in the nucleus of vascular endothelial cells of normal skin tissues and the 8-OHdG expression was negative (Figure 3C).

The results of image analysis showed that there was significant difference in the value of average light absorbance and the positive area rate of 8-OHdG expression between the proliferative stage of hemangioma and the involuting stage of hemangioma, the normal skin tissue (P<0.05). There was no significant difference between the involuting stage of hemangioma and the normal skin tissue (P>0.05) (Table 1).

The expression of 4-HNE

A large number of brown-yellow particles were found in the nucleus of the endothelial cells and the expression of 4-HNE was positive in the proliferative stage (Figure 4A). A small amount of brown-yellow particles were found in the nucleus of the endothelial cells and the expression of 4-HNE was negative in the involuting stage (Figure 4B). There is a minimal amount of brown-yellow particles in the nucleus of vascular endothelial cells of normal skin tissues and the 4-HNE expression was weak (Figure 4C).

The results of image analysis showed that there was significant difference in the value of average light absorbance and the positive area rate of 4-HNE expression between the proliferative stage of hemangioma and the involuting stage of hemangioma, the normal skin tissue.
There was no significant difference between the involuting stage of hemangioma and the normal skin tissue (P>0.05) (Table 3).

The expression of 8-OHdG, 4-HNE and NTY detected by QDs-SA

We can clearly see orange-red light in the hemangioma and adjacent tissue labeled by quantum dot under the fluorescence microscope, which indirectly display the exiting site of 8-OHdG, 4-HNE and NTY. The background is clean and showed no nonspecific binding. The positively expression site of 8-OHdG, 4-HNE and NTY were mainly located in the nucleus of vascular endothelial cells of hemangioma and adjacent tissues. In the nucleus of endothelial cells of proliferative hemangioma showing strong red fluorescence, which showed high intensity (P<0.05). There was no significant difference between the involuting stage of hemangioma and the normal skin tissue (P>0.05) (Table 2).

The expression of NTY

A large number of brown-yellow particles were found in the nucleus of the endothelial cells and the expression of NTY was positive in the proliferative stage (Figure 5A). A small amount of brown-yellow particles were found in the nucleus of the endothelial cells and the expression of NTY was negative in the involuting stage (Figure 5B). There is a minimal amount of brown-yellow particles in the nucleus of vascular endothelial cells of normal skin tissues and the NTY expression was very weak (Figure 5C).

The results of image analysis showed that there was significant difference in the value of average light absorbance and the positive area rate of NTY expression between the proliferative stage of hemangioma and the involuting stage of hemangioma, the normal skin tissue (P<0.05). There was no significant difference between the involuting stage of hemangioma and the normal skin tissue (P>0.05) (Table 3).

Table 2. Light absorbance value and positive reaction area ratio of 4-HNE expression in the proliferative and involuting stage of hemangioma and normal skin tissue (X ± s)

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of cases</th>
<th>Mean absorbance</th>
<th>Rate of positive area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferative stage</td>
<td>10</td>
<td>0.2954 ± 0.0602</td>
<td>▲ 0.2985 ± 0.0598</td>
</tr>
<tr>
<td>Involuting stage</td>
<td>18</td>
<td>0.0957 ± 0.0105</td>
<td>0.985 ± 0.0115</td>
</tr>
<tr>
<td>Normal skin</td>
<td>5</td>
<td>0.0913 ± 0.0115</td>
<td>0.925 ± 0.0121</td>
</tr>
</tbody>
</table>

*Comparing with involuting hemangioma and normal skin tissue, ▲ P<0.05.
Table 3. Light absorbance value and positive reaction area ratio of NTY expression in the proliferative and involuting stage of hemangioma and normal skin tissue (x̄ ± s)

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of cases</th>
<th>Mean absorbance</th>
<th>Rate of positive area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferative stage</td>
<td>10</td>
<td>0.3253 ± 0.0640</td>
<td>0.3158 ± 0.0621</td>
</tr>
<tr>
<td>Involuting stage</td>
<td>18</td>
<td>0.0954 ± 0.0273</td>
<td>0.0967 ± 0.0297</td>
</tr>
<tr>
<td>Normal skin</td>
<td>5</td>
<td>0.0847 ± 0.0264</td>
<td>0.0856 ± 0.0267</td>
</tr>
</tbody>
</table>

*Comparing with involuting hemangioma and normal skin tissue, *P<0.05.

expression of 8-OHdG, 4-HNE and NTY (Figure 6A-C). A small amount of red fluorescence was observed in the nucleus of the endothelial cells of the involuting hemangioma, and the expression of 8-OHdG, 4-HNE and NTY was weak (Figure 7A-C). A minimal amount of or no red fluorescence was observed in the nucleus of the endothelial cells of the normal skin tissues, and the expression of 8-OHdG, 4-HNE and NTY was weak, which indirectly showed the presence of 8-OHdG, 4-HNE and NTY (Figure 8A-C).

Discussion

Skin hemangioma is a common benign vascular tumor in infants and the abnormal proliferative of vascular endothelial cells is the main feature, but the exact mechanism of its pathological evolution is still not very clear. Vascular endothelial cells play an important role in the occurrence and development of hemangioma, while vascular endothelial cells are regulated by a series of positive and negative regulators. It is generally believed that the proliferation and regression of vascular endothelial cells are the key reason for the growth and regression of hemangioma. The proliferation of vascular endothelial cells and angiogenesis are regarded as the key link [5, 6].

Cell apoptosis is a process of active death of cells for multicellular organisms regulated by gene, which regulates the development and maintain internal environment stability [7]. It is the cell that activates the enzyme activity, such as endogenous DNA enzyme, through the endogenous pathway and a series of signal cascade reaction under certain conditions and induces natural cell death. Oxidative damage caused by oxidative stress is an important factor to cause a variety of injury and pathology of the human body [8].

DNA oxidative damage induced by reactive oxygen species (ROS) is an important field in the study of the mechanism of carcinogenesis, and DNA oxidative damage is closely related to aging, cancer, cardiovascular disease, chronic inflammation and other diseases. This damage can be induced by various factors, including endogenous metabolites, chemicals, drugs, smoking, ionizing and non ionizing radiation, etc. [9]. The mechanism is mainly related to the generation of ROS. There are many ways of DNA repair to eliminate damage, in order to maintain genome stability, thus preventing the occurrence of disease. Too much ROS can contribute to the damage of human normal cells and tissues because the balance between oxidation and antioxidation is destroyed [10, 11]. The ROS directly attack the biological macromolecules DNA or RNA and induce oxidative damage, resulting in the formation of oxidative modification products, which can affect the replication process of DNA and lead to the mutation of cancer suppressor and promoting gene, and then inhibit cell apoptosis, and lead to proliferation, angiogenesis and genomic instability.

ROS is the most important free radicals in the body. It can lead to oxidative damage of large biological molecules such as DNA, lipid and protein, generate 8-OHdG, 4-HNE and NTY, cause changes in cell function, and ultimately lead to the decline of tissue and organ function. Therefore, the content of 8-OHdG, 4-HNE and NTY can represent the severity of oxidative damage in cells, which are the recognized markers of oxidative damage [12, 13].

8-OHdG production id due to the result of the oxidation of guanine, and guanine is a component of DNA. Hydroxyl radical can directly act on the formation of 8-OHdG. The hydroxyl radicals directly or indirectly produced by various physical and chemical factors act on deoxyguanylic acid (dGMP) and cause the increased expression of 8-OHdG [14]. A large number of studies have indicated that the oxidative damage of DNA is closely related to the occurrence of tumor. 8-OHdG is the most important type of oxidative damage in DNA. 8-OHdG can be used as a biological marker to study the mechanism of DNA oxidative damage in tumor [15].

The quantum dot is a semiconductor nanocrystals, and the size is generally between 1-100
Hemangioma etiology

At present, the quantum dot has been developed as a new fluorescent marker. Compared with traditional markers, quantum dot have the following characteristics: (1) High fluorescence intensity and high sensitivity; (2) Wide range of fluorescence spectrum; (3) One excitation source can generate different emission

Figure 6. Expression of 8-OHdG in proliferative, involuting hemangioma and normal skin tissues (QDs-SA staining, ×200). A. Strong red fluorescence could be observed in the nucleus of the endothelial cells of the proliferative hemangioma and the expression of 8-OHdG was high; B. A small amount of red fluorescence could be observed in the nucleus of the endothelial cells of the proliferative hemangioma and the expression of 8-OHdG was weak; C. A minimal amount of or no red fluorescence was observed in the nucleus of the endothelial cells of the normal skin tissues, and the expression of 8-OHdG was very weak.

Figure 7. Expression of 4-HNE in proliferative, involuting hemangioma and normal skin tissues (QDs-SA staining, ×200). A. Strong red fluorescence could be observed in the nucleus of the endothelial cells of the proliferative hemangioma and the expression of 4-HNE was high; B. A small amount of red fluorescence could be observed in the nucleus of the endothelial cells of the proliferative hemangioma and the expression of 4-HNE was weak; C. A minimal amount of or no red fluorescence was observed in the nucleus of the endothelial cells of the normal skin tissues, and the expression of 4-HNE was very weak.
wavelengths; (4) Light bleaching is well tolerated; (5) Generally non-toxic; (6) The surface can be modified by a variety of genes and is easy to achieve kinds of tags. The quantum dot is an important material foundation in the field of molecular imaging, and it has shown a good prospect in the field of biological imaging and molecular marker technology. With the integration of multi spectral imaging analysis technology and the location feature of optical imaging technology, the quantum dot can provide the distribution curve of various chemical or biochemical composition in the sample, and obtain qualitative, quantitative and positioning analysis information. Therefore, based on the multi spectral imaging analysis technology of quantum dot labeled molecular probe, quantum dots are expected to break through technical barriers and showed a variety of ingredients in tumor tissues with high sensitivity and high specificity [16-19].

This experiment used quantum dot staining to quantitative detects the expression level of 8-OHdG, 4-HNE and NTY in different stages of hemangioma and normal skin tissues adjacent to the tumor. Statistical analysis showed that the expression of 8-OHdG, 4-HNE and NTY was statistically significantly higher in proliferative hemangioma than in involuting stage and normal skin tissues (P<0.05), while there was no significant difference in the expression of 8-OHdG, 4-HNE and NTY in the involuting hemangioma and normal skin tissues (P>0.05). The immunohistochemistry results also showed that the free radical oxidative damage products, 8-OHdG, 4-HNE and NTY, in hemangioma mainly existed in vascular endothelial cells and highly expressed in proliferative stage and lowly in involuting hemangioma or normal skin tissues. Suguira's [20] and other studies have found that the increase of reactive oxygen species is closely related to the damage of vascular endothelial cells. The largely increased content of 8-OHdG, 4-HNE and NTY makes the oxidative damage of the hemangioma tissues increased, and ultimately makes the hemangioma continue to hyperplasia, which means the oxidative damage of DNA plays an important role in the occurrence of hemangioma. The oxidative damage initiating cell carcinogenesis also related to the inhibition of apoptosis and promotion of cell proliferation. But, the specific mechanism of 8-OHdG, 4-HNE and NTY in the development of hemangioma has not been studied clearly and is needed further discussion. DNA oxidative damage may be a common feature of carcinogenesis. 8-OHdG, 4-HNE and NTY are a kind of early effect markers which...
reflect the oxidative damage of DNA. 8-OHdG, 4-HNE and NTY, as biomarkers of DNA oxidative damage by endogenous and exogenous factors, are a very promising index. Detection of 8-OHdG, 4-HNE and NTY can be used to evaluate the effect of antioxidants on the DNA oxidative damage. Thus, blocking DNA oxidative damage, improving the vitality of repair enzymes of the DNA oxidative damage, reducing the production of 8-OHdG, 4-HNE and NTY, and reducing the accumulation of gene mutations in body, can reduce the possibility of suffering from cancer and impel the hemangioma regress, so as to achieve the purpose of the treatment of hemangioma.

The results above suggest that DNA oxidative damage represented by 8-OHdG plays an important role in the occurrence and progression in hemangioma. The base mispairing caused by the formation of 8-OHdG is a major cause of gene mutation. Therefore, the progression of hemangioma may be related to the persistent DNA oxidative damage in the cells.

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Disclosure of conflict of interest

None.

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Hemangioma etiology

