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Tissue and serum mRNA profile of MMPs-2/9 as a potential novel biomarker for the most individual approach in infantile hemangiomas and cancer disease

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ABSTRACT

Propranolol is a widely-known beta-blocker approved for treating infantile hemangiomas (IH). The mechanisms behind the spectacular IH involution after propranolol treatment remain unclear. Recently, there is strong evidence of overexpression of numerous angiogenic factors in IH tissues, and it is reported that propranolol influences their pathways. However, a number of MMPs studies is highly limited. Here, for the first time, we propose a comprehensive approach by analyzing the expression levels of metalloproteinases-2/9 (MMPs-2/9) and tissue metalloproteinase inhibitor-2 (TIMP-2) *in vivo* on both, molecular and immunohistochemical levels, and in both, IH tissues and in the serum of IH patients, and relates the obtained results to the tumor's biology and systemic propranolol treatment.

Material and methods: MMPs-2/9 and TIMP-2 were analyzed in 71 IH tissue samples using immunohistochemistry and real-time PCR, and in 50 serum samples of IH patients by ELISA.

Results: Significantly lower MMPs-2/9 and higher TIMP-2 levels were observed in IH tissues on the mRNA level as well as lower serum MMP-2 concentration among the treated individuals.

Conclusion: MMPs-2/9 and TIMP-2 are both involved in the biology of IH and the propranolol pathways enabling their antiangiogenic properties. The most reliable method of IH examination appears to be direct MMPs-2/9 mRNA evaluation in tumor tissue; and MMP-2 evaluation in patients' serum is a valuable complement to it. Tissue and serum mRNA MMPs assessment may represent a suitable novel biomarker identifying tumor progression and involution processes with potential clinical impact in IH as well as in cancer disease.

1. Introduction

Although infantile hemangiomas (IH) are the most common tumors of early childhood, their biology is still unrecognized. Most lesions are not apparent at birth. The incidence rate of all newborns is 1.1–2.6% but may affect up to 10% of all infants (Willenberg and Baumgartner, 2008; Chang et al., 2008; Schwartz et al., 2010). IH are composed of endothelial cells mixed with pericytes, dendritic cells and mastocytes and present a positive stain with GLUT-1 (glucose transporter-1) glycoprotein in all stages of development. IH show an unique mechanisms of reversible proliferation leading to division into proliferation and involution phases, and two opposing theories of IH pathogenesis have been given (Yu et al., 2004). The proliferation phase of IH is characterized by rapid growth and blood accumulation in tumor mass and usually lasts to the end of the first year of life (Khan et al., 2008). The process of IH involution is long and generally unknown, taking months to even several years (Chang et al., 2008; Boscolo and Bischoff, 2009; Itinteang and Withers et al., 2014). Age is a widely-accepted criterion of developmental phases; however, in some IH types or locations, for example the parotid gland or lip, the proliferation phase may persist until the end of the second year of life (Clemis et al., 1975).

Distinct differences can be seen in the microscope images of the two IH phases. During proliferation, IH are composed of densely-packed capillaries composed of endothelial cells surrounded by pericytes. Frequent mitoses are the morphological sign of the high cellular proliferation rate. At the time of involution the capillaries are progressively replaced with fibrous and fatty tissue (Mulliken and Glowacki, 1982; Enjolras and Mulliken, 1993).

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The exact mechanisms of IH pathogenesis is currently highly investigated due to the fact that it reflects defects in angiogenesis. Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are known to be consistently expressed in IH tissues and both have been shown to play an important role in IH biology (Takahashi et al., 1994; Zhang et al., 2005). However, 20 pro-angiogenic factors other than VEGF are known to be present, the levels of which increase in patients with IH (Richter and Friedman, 2012).

Matrix metalloproteinases (MMPs) are much less investigated in IH. The MMP family consists of protein and peptide hydrolases and includes MMP-1, MMP-2, MMP-3, MMP-9, and MMP-10, which play major roles in ECM degradation by hydrolyzing the basement membrane collagen (Tallant et al., 2010; Visse and Nagase, 2003). Among them, MMP-2 (72 kd gelatinase A) and MMP-9 (92 kd gelatinase B), which degrade collagen types IV, V, and X, act together with growth factors, cytokines, and growth factor binding proteins (Salamonsen, 1996; Chang and Werb, 2001; McCawley and Matrisian, 2001). The activation of pro-MMPs is inhibited by tissue inhibitors of metalloproteinases (TIMPs), and both MMPs and TIMPs play an important part in many physiological and pathological conditions, including neoplasms (Salamonsen, 1996; Salmela et al., 2001). Recently MMPs-2/9 presence was confirmed in urine of IH patients (Kleber et al., 2012) and MMP2 in hemangioma tissue (Zhong et al., 2009).

Up to 20% of children with IH require therapy (due to severe hemangiomas); however, the treatment may be challenging. Glucocorticoids, interferon alpha and cyclophosphamide previously used in these cases evoke serious side effects (Barlow et al., 1998). Propranolol has been demonstrated to be extremely effective in reducing infantile hemangiomas. While other beta-blockers are also used in IH treatment it is known from literature that results are less spectacular (Chinnadurai et al., 2016; Tasani et al., 2017)

Propranolol has currently become the standard of care despite the fact that its influence on IH tissue is not clearly understood (Léauté-Labrèze et al., 2008; Léauté-Labrèze et al., 2015; Hoeger et al., 2015 Jul). Considering the above, this is, to the best of our knowledge, the first complex study to use immunohistochemistry and mRNA analysis to determine the spectrum of MMPs-2/9 and TIMP-2 in both types of available materials, i.e. directly in IH tissue and in the serum of IH patients. This approach allows the different levels and aspects of the biology of examined tumors to be integrated with regard to the influence of propranolol treatment processes, and to reveal its expected clinical impact in IH and potentially in other tumors. The present retrospective study was carried out as the part of the ‘Mechanism of propranolol action and pharmacogenetics in infantile hemangiomas’ project realized with a grant of The Ministry of Science of Poland (No N407691340) and in compliance with the agreement of The Bioethics Committee of The Medical University of Lodz, Poland (No RNN/209/09/KE).

2. Materials and methods

All the treated patients were elected for systemic therapy due to physiological impairment or serious cosmetic defect. Size of hemangioma ranged from 1 to 12 cm (mean 3,7 cm) on the beginning of therapy. The most common IH location was the face with 38 cases (53.5%); the total for head and neck was n = 50 (70.42%), followed by the chest (n = 5: 7.04%), trunk and extremities (n = 16: 22.54%) and almost all lesions were focal and superficial with subcutaneous component, no segmental IH were included in study group. No other pathologies except from hemangioma were diagnosed in our patients. There were no premature children born before 38 Hbd among IH patients.

Exclusion criteria in our studies comprised age below four weeks, clinical signs of involution of the tumor and previous treatment with steroids. Steroids were found to be moderately effective in many studies assessing size and volume reduction comparing to propranolol and they had evoked important side effects.

The treatment with propranolol in our studies was initiated after standard procedures: biochemical tests, EKG, ECHO and cardiologist consultation. Initial doses were 1 mg/kg/day rising to 2 mg/kg/day under heart rate and blood pressure control. The duration of propranolol treatment was 5–12 months (mean 7.93, SD ± 3.70, median 9.00, IQR 5.0-12.0). There was no other beta-blocker use among our patients to avoid misinterpretation of results reflected the main goal of research project.

2.1. Direct IH tissues studies

Routinely fixed in 10% buffered formalin, paraffin embedded 71 infantile hemangiomas tissue sections were selected for our study. The tumor tissues were obtained from 54 girls (76.1%) and 17 boys (23.9%) aged from one month to 60 months (Mean 25.5, SD ± 15.6). The slides about the thickness 3–4 of micrometers where prepared from tumor tissue and stained with hematoxylin and eosin (H & E). All the cases were reviewed routinely by two pathologists and confirmed by immunochistrochemistry Glut-1(+). The growth phases of hemangiomas are accepted as that of depicted in Mulliken and Enjolras' description (Mulliken and Enjolras, 2004).

2.1.1. Immunohistochemistry

The mouse monoclonal antibodies MMP-2, MMP-9 and TIMP-2 (Novocastra, UK) and the Envision system (DAKO, Glostrup, Denmark) were used for immunohistochemical studies (IHC). Standard target retrieval procedures were performed: MMP-2 (dilution 1:40) – water bath, pH 9.0; MMP-9 (1:40) – water bath, pH 9.0 and TIMP-2 (1:20) – high pH. The expression of the investigated proteins was estimated with a computer image analysis system (Multi Scan Base v. 8.08 – Computer Scanning System, Ltd.). The positive reaction, observed as a brown coloration of the cell cytoplasm, and the results of estimation of 1000 tumor cells were shown as an index (%).

2.1.2. PCR mRNA

Total cellular RNA was isolated from paraffin-embedded IH tissues according to Chomczynski’s method. The obtained RNA was frozen and kept at −70 °C until analysis. The expression of cytokines was assessed by RT-PCR. A two-step procedure was applied: firstly DNA was synthesized on an mRNA matrix isolated previously from hemangiomas and than the standard polymerase real time chain reaction was performed.

2.2. IH patient serum studies

Serum samples were collected from IH patients prior to the first dose of propranolol and three months after continuous therapy. The patients were 39 girls and 11 boys aged 1–15 months (Mean 4.77, SD ± 3.37), with tumors in the proliferation phase of development, selected for propranolol treatment according to the criteria described above. The shortest diameter of tumor mass was 1 cm. The serum samples were separated within one hour of sampling and kept frozen in −20 °C until analysis. MMPs-2/9 and TIMP-2 levels were determined with the use of commercial antibody sets Quantikine ELISA kits (R & D Systems Inc., MN, USA) according to the manufacturer’s instructions.

2.3. Statistical methods

Descriptive statistics were presented as mean with standard deviation (SD) and as median with interquartile range (IQR). The distribution of quantitative variables was assessed by the Shapiro-Wilk test: As the distributions were not found to be normal, nonparametric methods of analysis were applied. Wilcoxon’s test for paired comparisons and the Mann-Whitney *U* test for non-paired continuous variables were used. Correlation coefficients were calculated with Spearman’s rank test. The