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The Role of Endoglin in Hemangioma

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Abstract:

Hemangiomas are the most common soft-tissue tumors of infancy, occurring in approximately 5 to 10 percent of one-year-old children. There is no universally accepted model for the etiology of hemangiomas, but there are many theories about the origin of hemangiomas. The purpose of this study was to describe the role of endoglin in hemangioma.

Materials and Methods:

We used human endoglin/CD105 Enzyme linked immunosorbent assay (ELISA) kit to examine serum endoglin concentrations of 30 patients with hemangioma.

Result: The serum endoglin concentrations of patients with hemangioma were significantly higher than control group.

Conclusion: Endoglin may have a role in the pathogenesis of hemangioma.

Keywords: Hemangioma, Enzyme linked immunosorbent assay, Endoglin

Introduction

Hemangiomas are the most common soft-tissue tumors of infancy, occurring in approximately 5 to 10 percent of one-year-old children, Mulliken and Glowacki [1,2]defined hemangiomas as vascular tumors with a growth phase, marked by endothelial proliferation and hypercellularity, and an involutional phase marked by decrease in cellularity.

The incidence of hemangiomas is 3 times higher in female than male Infants. There is also an increased occurrence in premature infants. Hemangiomas usually appear soon after birth (though up to 30% may be present at birth), typically proliferate during the first year of life and then involute during the childhood years (up to 12 years). The terms capillary and cavernous hemangioma are out of date and the lesions are more appropriately described according to the depth of the lesion as superficial, deep, and compound hemangioma. Superficial hemangiomas originate from the papillary dermis and present as bright red macular or papular masses (previously called capillary or strawberry hemangioma). Deep hemangiomas originate from the reticular dermis or subcutaneous tissues and appear as bluish or relatively colorless masses (previously called cavernous hemangioma). Compound hemangiomas have superficial and deep components and were previously called capillary cavernous hemangiomas. Hemangioma can be complicated by ulceration, infection, hemorrhage and residual deformity. [1,3,4]

The histological features are dependent on the stage of the lesion, in the proliferative phase, the lesion is highly cellular and contains plump proliferating endothelial cells and pericytes, with a high mitotic activity and numerous mast cells, vascular channels are not prominent. In the involutive phase, the endothelial cells are flattened, the cell turnover is normal and there are few mast cells, vascular channels filled with blood cells predominate, and the lesion is eventually replaced by fibrofatty tissue. [1]

There's no universally accepted model for the etiology of hemangiomas, but there are many theories about the origin of hemangiomas. It has been suggested that some hemangiomas originate from a first-trimester developmental error in vasculogenesis, others says that the increased incidence of hemangiomas in children born to women post-chorionic villus sampling suggests hemangiomas are of placental origin. In this sampling procedure, a fetal trophoblast sample is surgically removed from the placenta, leading to placental embolization and maternal fetal transfusion. Some studies consider that underdeveloped vasculature in the embryo could also result in vascular endothelial nests that proliferate faster after birth than normal blood vessels. Others consider that rapid tumor growth in the postnatal phase is possibly a result of the loss of angiogenic inhibitors from the placenta or mother. Others suggesting that the abnormal growth seen in proliferating hemangiomas are related more to the local release of growth factors than to an altered endothelial phenotype.[5]

Today, the following treatments are used for hemangiomas: corticosteroids, intralesional interferon alpha, imiquimod, vincristine, variety of lasers, debulking surgery and watchful waiting. These treatments can be administered individually or in combination.[3]

Endoglin is an auxiliary receptor for Transforming growth factor beta (TGF-β) involved in the interaction with type II receptors for both TGF-β and activin. Endoglin counteracts the inhibitory effect of TGF-β on cell proliferation in several cell types including endothelial cells In this respect, it is of interest that the inhibition of endoglin expression enhanced the ability of TGF-β1 to suppress growth, migration and capacity to form capillary tubes of cultured endothelial cells. In the absence of TGF-β1, endoglin shows an anti-apoptotic effect in endothelial cells under hypoxic stress, suggesting a protective role for endoglin against pro-apoptotic factors. As endoglin directly interacts with a variety of TGF-β type I receptor, this raises the possibility for additive or opposing effects of endoglin on TGF-β receptor signaling. Thus, endoglin shows an inhibitory effect on TGF-β / Activin receptor-like kinase 5(ALK5)/ Smooth muscle acting receptor3 (Smad3) cellular responses which block endothelial cell proliferation and enhance extracellular matrix deposition. In addition,

endoglin may be required for TGF-β1/ALK1 signaling in some cell types, especially endothelial cells inducing endothelial cell proliferation and degradation of extracellular matrix. This balance between ALK5 and ALK1 may play a role in the regulation of cell growth and differentiation in cells that express endoglin as well as ALK1 and ALK5 . The mechanism by which endoglin potentiates TGF-β/ALK1 signaling appears to involve direct association of ALK1 with the cytosolic and extracellular domains of endoglin, with the extracellular domain1 mediating the enhancement of ALK1 signaling. These studies suggest that the functional association of endoglin with ALK1 is critical for endothelial cell responses to TGF-β.(6-10)

Patients And Methods

Patients

This study was done on 30 patients who attended the pediatric surgery and dermatology outpatient clinics, Alexandria university hospitals and proved by examination to have hemangiomas. Twelve patients had capillary (superficial) hemangioma **(Fig 1)**, 18 patients had cavernous hemangiomas of which 9 patients had deep **(Fig 2)** and 9 patients had compound type **(Fig 3)**

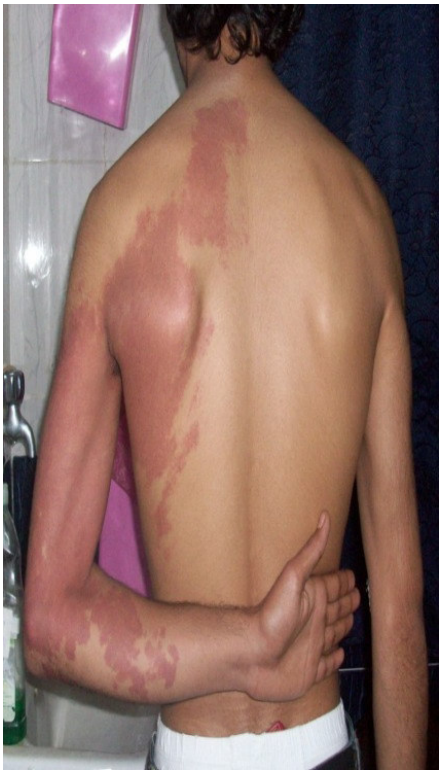

Fig 1: Superficial hemangioma (capillary).

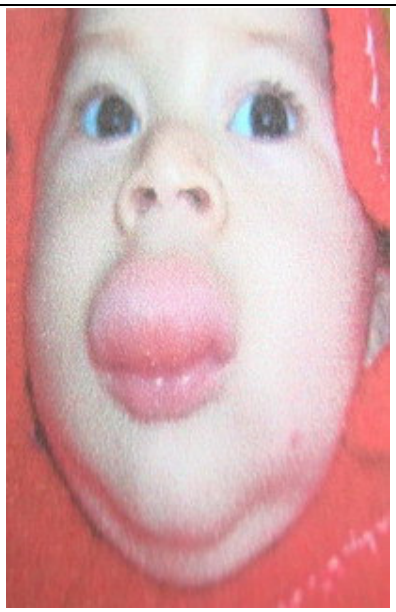

Fig 2: Deep hemangioma (cavernous).

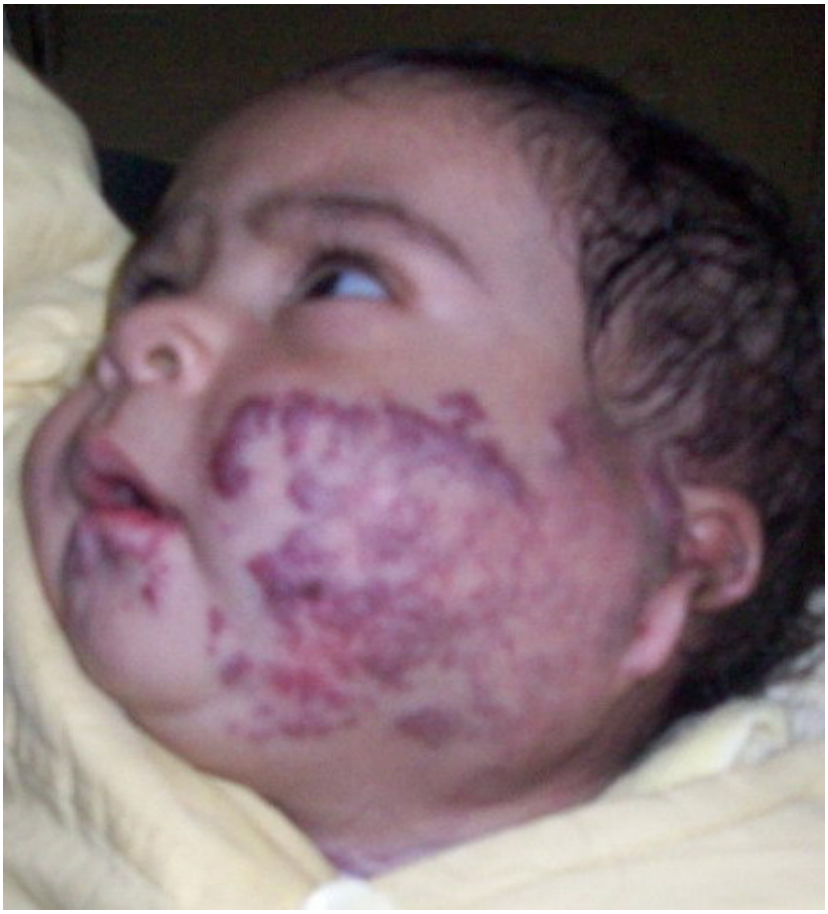

Fig 3: Compound hemangioma (capillary cavernous).

Twenty patients were females and 10 patients were males. Their age ranged from 2 months up to 30 years.

The patients or their parents complaints were :

- Disfiguring patch in14 patients ,
- Non-painful swelling in 9 patients ,
- Painful swelling in 3 patients ,
- Bleeding lesion in 3 patients,
- Itchy lesion in one patient.

-The lesions dated since birth (0) up to 30 years with a mean duration of 7.83 years and a median duration of 2 years.

- Hemangiomas were reported to be gradually increasing in size in 63.3%, stationary in (20.0%) and regressive in 16.7% of patients.

- Ten percent (10.0%) of the parents of the studied patients were married to relatives and 90.0% were non-consanguineous.

- The majority of cases (80.1%) had uncomplicated hemangiomas and 19.9% reported complications such as bleeding, crustation or infection.

- Hemangiomas were located in the face in (36.7%), in the hand in 10.0% and in the anterior chest wall or the scalp in 6.7% of patients. Others sites, such as the arm, the abdominal wall, the labia majora or the buttock were reported in 3.3%. Multiple locations of hemangiomas were encountered in 26.7% of the studied patients.

- Hemangiomas measured from 0.5-40 cm. (mean= 4.810) in width and 0.5-40 cm. (mean=2 cm.) in length.

Control group: 60 subjects (age & sex matched with the patients with hemangiomas) suffering from minor dermatological problems other than hemangioma or admitted to pediatric surgical department for minor surgery (free from hemangioma)

Venous blood sample were obtained from both groups after signing an informed consent form (Informed consent was taken from the parents of children patients participating in the study), The study was approved by the Alexandria Faculty of Medicine, Research Ethics Committee.

Methods

All patients were subjected to:

1-Complete history taking

2-Clinical examination

3-Photography:

The lesions were photographed by a Kodak camera with resolution 8 mega pixel.

4-Investigation:

Venous blood samples (3-5 ml) were collected in tubes and allow them to clot for 30 minutes before centrifugation for 15 minutes at approximately 1000 x g, then we remove serum and store samples at -20° C.

We used human endoglin/CD105 ELISA kit [11], which includes the following reagents:

Endoglin microplate: 96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against human endoglin.

Endoglin conjugate: 21 mL of mouse monoclonal antibody against human endoglin conjugated to horseradish peroxidase with preservatives.

Endoglin standard: 100 ng of recombinant human endoglin in a buffered protein base with preservatives.

Assay diluent: 11 mL of a buffered protein base with preservatives.

Calibrator diluent: 21 mL of a buffered protein base with preservatives.

Wash buffer concentrate: 21 mL of a 25-fold concentrated solution of buffered surfactant with preservatives.

Color reagent A: 12.5 mL of stabilized hydrogen peroxide.

Color reagent B: 12.5 mL of stabilized chromogen (tetramethylbenzidine).

Stop solution: 6 mL of 2 N sulfuric acid.

Plate covers: 4 adhesive strips.

Procedure:

All reagents were brought to room temperature before its use then diluted 20 mL of wash buffer concentrate with distilled water to prepare 500 mL of wash buffer.

Color reagents A and B were mixed together in an equal volumes then 200 micro liter of the resultant mixture was required per well.

Endoglin standard was reconstituted with 1.0 mL of distilled water. This reconstitution produces a stock solution of 100 ng. /mL, and then mixed the standard to ensure complete reconstitution and allowed the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

We pipetted 900 micro Liter of calibrator diluent into the 10 ng/mL tubes, and then we pipette 500 micro Liter into the remaining tubes. We used the stock solution to produce a dilution series. The 10 ng/mL standard serves as the high standard and the calibrator diluent serve as the zero standard (0 ng/ml). We added 100 micro Liter of assay diluent to each well and then we added 50 micro Liter of standard control or sample per well and cover it with the adhesive strip provided and incubated it for 2 hours at room temperature on a horizontal orbital microplate shaker. A plate layout was provided to record standards and samples assayed.

We aspirated each well and wash, repeating the process three times for a total of four washes.

We wash by filling each well with wash buffer (400 micro liter) using autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, we removed any remaining wash buffer by aspiration. We inverted the plate and blot it against clean paper towels.

Endoglin conjugate was added 200 micro L to each well and covering with a new adhesive strip then incubated for 2 hours at room temperature on the shaker.

We repeat the aspiration/wash as mentioned above.

Then we added 200 micro L of substrate solution to each well and incubated for 30 minutes at room temperature on the benchtop with protection from light.

We added 50 micro L of stop solution to each well. The color in the wells changed from blue to yellow.

Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm

Data Processing and Analysis

After data collection, raw data was coded and scored and a coding instruction manual was prepared. Data were fed to the computer using Epi-Info (version 3.0) and statistical analysis was performed using Statistical Package for Social Sciences (SPSS version 15.0). Roc analysis was performed using Med calc software. Significance of the obtained results was judged at the 5 % level.

1-Data processing:

Complete confidentiality was maintained while the data were being processed. This stage had two major objectives:

1- Clean data by performing a series of comprehensive checks, making corrections whenever possible. Different statistical procedures (frequencies, means, standard deviations, median, and Interquartile Range (IQR) and cross tabulations) were used to check the validity of data and spot any error.

2- Produce analytic results, which involved the recording of variables into forms required for analysis.

2-Data analysis:

The following statistical measures were used:

1- Descriptive Statistics such as frequency, distribution, mean, median, standard deviation and interquartile range to describe selected socio-demographic and clinical profile of patients with hemangioma as well as their endoglin level.

2- Bivariate analysis; student t test and One Way Anova (F) tests of significance were used for statistical association between patients' profile and their endoglin level.

3- Multivariate analysis was performed to assess the joint contribution of selected socio-demographic and clinical variables (independent variables) and endoglin level (Dependent variable). Variables included in the multiple linear regression model were age (years), sex (male = 0, female = 1), onset (other = 0, since birth = 1), type , location (others = 0, head = 1) , course (others = 0, aggressive = 1), size (Others = 0, large = 1), complication (no = 0, yes = 1), and type (others = 0, cavernous = 1).

4- ROC curve analysis was performed to evaluate the diagnostic accuracy of endoglin level for the overall group of patients with hemangioma. A ROC curve is a graphical representation of the tradeoffs between sensitivity and specificity. ROC curve analysis was performed with use of data related to endoglin level (ng/ml) from 30 hemangioma patients, and 60 controls.

Results

The comparison of endoglin level between cases of hemangioma and their controls showed that The mean endoglin level for the cases was 8.99 1.55 ng/ml and this was significantly higher than that for their controls (7.13 2.21 ng/ml) where t = 4.961, and p = 0.000 .

The relation between endoglin level and selected demographic and clinical factors.

Spearman correlation between age of the studied patients and their endoglin level revealed a statistically significant negative correlation between age of the patients and endoglin level (r = -0.348, p = 0.030). This means the younger the age of patients with hemangioma is, the more increase in endoglin level will be.

Also a statistically significant negative correlation between duration of hemangioma and the endoglin level was found (r = - 0.356 and p = 0.027). This means the shorter the duration of the lesion is the more increase in the endoglin level will be. However, no statistical significant correlations were noted between endoglin level and either onset or size of hemangioma lesion (p = 0.104 and 0.080 respectively) .

In the present study despite that the endoglin level of male patients was higher than that of female patients (9.40 1.78 versus 8.79 1.43 ng/ml), yet this difference was not statistically significant (t = 1.017, p = 0.318) .

Moreover, the association between endoglin level of the studied patients and type of hemangioma was illustrated showing that the mean endoglin level for patients with cavernous hemangioma (9.91 1.17 ng/ml) was higher than that for those patients with capillary type (6.71 1.86 ng/ml) or that for patients with combined hemangiomas (8.86 1.04 ng/ml). However, the difference was not statistically significant (F = 2.771, p = 0.080).

Also, the mean endoglin level for patients with complications (9.74 0.57 ng/ml) was higher than that for those patients without complications (7.96 1.48 ng/ml). However, the difference was not statistically significant (t = 2.015, p = 0.292).

ROC analysis

The diagnostic accuracy of endoglin level was evaluated for the overall series using ROC curve analysis. A ROC curve is a graphical representation of the tradeoffs between sensitivity and specificity. ROC curve analysis was performed with use of data related to endoglin level (ng/ml) from 30 hemangioma patients, and 60 controls. The ROC curve analysis suggested that the most useful cutoff value of serum endoglin concentration was the 8.4 ng/mL value, where the sum of sensitivity (73.33%) and specificity (70.00%) was the highest (**Fig 4** & **Table 1**).

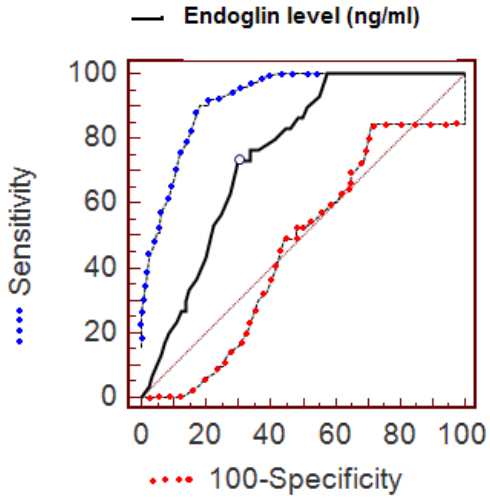

Fig 4: ROC curve of serum endoglin concentration for the diagnosis of hemangioma. (Sensitivity plotted against 100 – specificity at different concentrations of endoglin).

Area under the ROC curve (AUC)	0.754
Standard Error	0.0418
95% Confidence Interval	0.662 to 0.831
Z statistic	5.543
Significance level P (Area=0.5)	0.0001

Table 1 :Roc curve analysis of the studied sample (30 patients & 60 controls)

Multivariate analysis

Predictors of elevated endoglin level

Multiple linear regression models were performed to predict the significant contributing factors associated with elevated endoglin level among the studied cases of hemangioma. After adjustment for confounders, higher level of endoglin were significantly associated with younger age (b = - 2.823, p = 0.002), female gender (b = 3.145, p = 0.005), since birth dating lesion (b = 2.657, p = 0.002), and aggressive lesion (b = 3.525, p = 0.008). These variables explained 79.639 % of the variability in elevated endoglin level (R2 = 79.639%) indicating that the overall model was statistically significant where F = 7.324, p = 0.007.

Discussion

In the present study among the thirty patients included there were 20 females (66.7%) and 10 males (33.3%) with a ratio of 2:1. In Anand et al [12] their study was on 2398 patients the male to female ratio 1:2.3. In Shapour et al [13] their study was on 32 patients the female to male ratio 1.9:1. This was more or less similar to literature where female predominance reached a ratio of 3:1.

In the present study we measure the serum soluble endoglin level in 30 patients with hemangiomas by human endoglin/CD105 ELISA kit [11], we found that the mean endoglin level for the cases was 8.99 1.55 ng / ml which is significantly higher than their control(7.13 2.21 ng / ml) where t=4.961 and p=0.000. As endoglin is an auxiliary receptor for TGF-β involved in the interaction with type II receptors for both TGF-β and activin. Endoglin counteracts the inhibitory effect of TGF-β on cell proliferation in several cell types including endothelial cells. Many studies were done to prove the inhibitory effect of TGF-β on Basic fibroblast growth factor (bFGF). Andrew et al [14] found in their study that TGF-β is a potent inhibitor of the proliferative activities of bFGF on vascular and capillary endothelial cells; this inhibition is of non competitive interaction and dose dependent. Mohammed et al [15] found that TGF-β inhibits bFGF induced proliferation of cultured bovine retinal endothelial capillary cells in dose dependent manner. Marijke et al [16] their study shows that the antagonism of fibroblast growth factor induced cell proliferation on bovine aortic endothelial cells in culture is of non competitive manner and dose dependent. Sakela et al [17] found that bFGF is potent inducer of angiogenesis in vivo and stimulate the production of both urokinase and tissue type plasminogen activators (PAs) in cultured bovine capillary

endothelial cells and this effect is diminished by pictogram amount of TGF-β, so TGF-β has opposing effect on bFGF induced proliferation on vascular and capillary endothelial cells. bFGF is critical to angiogenesis and this was proved by Zahng Rong et al[1] who studied the concentration of bFGF in 25 patients with proliferating and 14 patients with involuting hemangiomas and he found that the serum bFGF concentration of proliferating hemangioma were significantly higher than those of involuting one.

From all the above information we can conclude that endoglin inhibits TGF-β which inhibits bFGF, so endoglin indirectly activate bFGF leading to vascular and capillary endothelial cell proliferation. This may prove that our result can be documented. To our knowledge there is no similar study to ours to date.

In the present study we found that there is a statistically significant negative correlation between age of the patient and endoglin level showed by spearman correlation r=-0.348 and p=0.030 which means that the younger the age of the patient, the higher the endoglin level and this is coincident with the nature of hemangioma that is proliferative in early phases and involutive in late phases. Michelle et al [19] reported in his study on hereditary hemorrhagic telangiectasia (HHT) type1 that there is inverse correlation between age and level of plasma endoglin indicating that age is effect modifier highest in children and decrease progressively with age as the levels of plasma endoglin measured by ELIZA in 197 individuals with and without HHT show inverse correlation with age r=-0.22 and p<0.002.

As regard the duration of hemangioma and endoglin level there is negative correlation as r=-0.356 and p=0.027 which means that the shorter the duration of the lesion the more the endoglin concentration. All the above results can prove that endoglin has a role in hemangioma as hemangioma starting proliferative in early phases where we found endoglin concentration is high and becoming involutive in late phases where we found endoglin concentration is decreasing.

From the result of our study (to our knowledge this is the first study to be done on the role of endoglin in hemangioma); higher level of endoglin were significantly associated with younger age, female gender, lesion dating since birth, and aggressive lesion.

Conclusion

Endoglin may have a role in the pathogenesis of hemangioma through its involvement in TGF-β signaling during developmental angiogenesis.

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