

Growth Factors, Their Receptors, Neuropeptide-Containing Innervation, and Matrix Metalloproteinases in the Proximal and Distal Ends of the Esophagus in Children With Esophageal Atresia

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Summary. *Objective.* The pathogenesis of esophageal atresia (EA) remains unknown despite a relatively high incidence of this anomaly in population affecting 1 newborn per 3000 live births. The aim of this study was to examine the relative occurrence of growth factors, their receptors, neuropeptide-containing innervation, and tissue-degrading enzymes – matrix metalloproteinases – in the proximal and distal parts of the esophagus with EA.

Materials and Methods. A histopathological study was conducted on 15 patients with EA. Tissues were processed for NGFRp75, PGP 9.5, TGF- β , FGFR, VEGF, EGFR and MMP-2 by means of biotin-streptavidin immunohistochemistry.

Results. In the control and EA-affected distal esophageal specimens, numerous and abundant NGFR-containing structures were detected, while in the proximal part of the esophagus, a decrease in their number was observed in patients. PGP 9.5 also marked neuronal structures similarly. TGF- β was found only in occasional cells in the EA-affected esophageal specimens, while control material demonstrated moderate to numerous TGF- β -containing structures. Abundance of FGFR and only occasional appearance of VEGF-positive cells were found in both the control and EA-affected material. A moderate number of connective tissue cells in controls contained EGFR. Compared with controls, the number of MMP-2 expressing cells in the EA-affected tissues was decreased in the proximal esophagus.

Conclusions. A decrease in PGP 9.5-containing neuronal structures in the proximal esophagus supports insufficient innervation of this part of the organ in EA. A decrease in MMP-2 positive cells in the esophageal atresia-affected proximal esophagus indicates also a possible decrease of tissue adaptive and regenerative reactions. Low expression of TGF- β and almost the absence of EGFR in the EA-affected specimens may result in disturbances of cell growth, proliferation, and differentiation, indicating a significant role of these substances in morphopathogenesis of EA. FGFR and VEGF seem not to characterize EA pathogenesis.

Introduction

Esophageal atresia (EA) is a rather frequent congenital disease affecting 1 per 3000 newborns (1). In Latvia, 41 newborns were operated on due to EA from 1996 to 2003 (2).

Rather often, EA is revealed together with congenital abnormalities of other organs and systems, such as heart, digestive system, reproductive system, excretory organs, as well as skeleton and nerve system. In older patients, gastroesophageal reflux is frequently observed. A study by Li et al. has shown the imbalance of neurotransmitter excretion in nerve vesicles, abnormal intrinsic dysplasia of nerve plexus, and increased expression of certain neuro-

peptides as the main characteristics of esophagus with abnormal intrinsic innervation, which may be responsible for the postoperative esophageal dysfunction in EA (3).

In histological studies, both muscle hypertrophy and chaotic organization of the esophagus blind end are the most frequently mentioned (4). Sometimes, the remnants of tracheobronchial wall, such as cartilage and altered mucoserous glands, are found in the distal end of the esophagus. Likewise the smooth muscle disorganization was observed (1) as well as disturbances of both blood supply and innervation.

However, morphopathogenesis of EA is still not

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understood at the molecular level. Within the last decade, much attention has been paid to the role of growth factors (5, 6), cell adhesion molecules (7), genes (8), and apoptosis (9) in the development of EA. In particular, the importance of bone morphogenetic protein (BMP) and sonic hedgehog protein (Shh) for the development of pathology has been investigated.

Despite the regulation of many functions in esophageal structures by neuropeptides, there are still incomplete data about the neuropeptide-containing innervation in EA. Protein gene product 9.5 (PGP 9.5) is a cytoplasmic neuron- and neuroendocrine cell-specific protein used to visualize the diffuse neuroendocrine system – neuropeptide-containing innervation (10). In healthy esophageal tissues, the stained nerve tissue forms a nearly complete ring, while in EA-affected tissues, neuronal elements are distributed in a form of isolated clusters of diffuse localization (11).

Fibroblast growth factor (FGF) affects cell proliferation, regulates cell growth and maturation (12) as well as embryogenesis (13).

Transforming growth factor β (TGF- β) controls the rates of cell growth and proliferation, stimulates cell maturation (14) as well as affects the formation of extracellular matrix (15). TGF- β is widely investigated in other esophageal anomalies and diseases.

Vascular endothelial growth factor (VEGF) stimulates endothelial proliferation, particularly under hypoxia (16).

Epidermal growth factor receptor (EGFR) is found in the tissues of epithelial, mesenchymal, and neuronal origin where they contribute to cell proliferation, differentiation, and maturation (17). Matrix metalloproteinases (MMPs) catalyze restructuring extracellular matrixes. They are the major proteolytic enzymes involved in extracellular matrix turnover. MMP-2 (gelatinase A) is one of the factors that cleaves type IV collagen – an important constituent of the basement membrane. MMPs play an important role in normal tissue homeostasis, but imbalance between those enzymes and their tissue inhibitors (TIMPs) is thought to be a critical factor in regulating tissue remodeling. MMP-2 is produced by fibroblasts, endothelial and epithelial cells, while MMP-9 – a second important proteolytic enzyme – is mainly produced by inflammatory cells (18).

Since morphopathological mechanisms of EA development are still obscure, the present study was aimed to investigate a relative occurrence of growth factors, their receptors, neuropeptide-containing innervation and tissue-degradating enzymes, MMPs, in the proximal and distal parts of the esophagus with EA.

Material and Methods

Material. The specimens were obtained from 15 newborns (9 boys and 6 girls). In 13 cases, the diagnosis of EA with distal tracheoesophageal fistula (TEF) was established, while 2 cases were found to have EA with proximal TEF. The newborns ranged in age from 21 hours to 1 month. In the control group, 12 specimens of the esophagus obtained from newborns whose cause of death was not related to gastrointestinal pathology were included. All tissue specimens were obtained in accordance with the ethical requirements of Riga Stradins University (state permission No. Iz2007/02/11).

Methods. *Biotin-Streptavidin Immunohistochemistry* (19). The tissue fragments were fixed for 24 hours in a mixture consisting of 2% formaldehyde and 0.2% picric acid in 0.1-M phosphate buffer (pH 7.2). The tissue fragments were washed for 12 hours in phosphate buffer (pH 7) containing 10% sucrose. Then tissues were embedded in paraffin, and the blocks of paraffinized tissues were sectioned into slides 6–7- μ m in thickness by means of a microtome. The slides were deparaffinized and prepared to detect the following growth factors and their receptors: NGFR p75 (code M3507, working dilution 1:150, Dako, USA), fibroblast growth factor receptor 1, FGFR1 (code ab10646, working dilution 1:100, Cambridge Science Park, UK); TGF- β (code ab1279, working dilution 1:1000, Cambridge Science Park, UK); VEGF (code M7273, working dilution 1:50, Dako, Denmark); epidermal growth factor receptors, EGFR (code M3563, working dilution 1:200, Dako, USA); innervation marker, PGP 9.5 (code Z5116, working dilution 1:200, Dako, Denmark); and tissue-degradating enzyme, MMP-2 (code AF902, working dilution 1:50, RD Systems, UK).

Histology. To obtain an overview picture, the slides were prepared from each material sample and stained with hematoxylin/eosin.

Quantification of Structures. For quantification of structures, the semiquantitative counting method was used. The designations were as follows: 0, negative reaction; 0/+, occasionally marked structures in the view field; +, a few positive structures in the view field; ++, a moderate number of marked structures in the view field; +++, a numerous number of marked structures in the view field; +++++, abundance of marked structures found in the view field (20, 21). The semiquantitative evaluation of specimens was performed by using a Leica DM R HC microscope (magnification \times 250).

Additionally, the nonparametric Kruskal-Wallis test was used to analyze the data (22).

Results

In the control specimens, NGFRs were detected in abundance in epithelial basal cells (Fig. 1), nerve

fiber bundles (Fig. 2), smooth muscle cells of small blood vessels as well as in nerve fibers around and in lymphatic tissues. NGFR expression was also abundant in Meissner's and Auerbach's nerve plexuses. In the EA-affected tissues, the similar pattern of NGFR distribution was observed in the distal esophagus (Fig. 3), even though the nerve plexuses were discerned only in some specimens because of deranged and chaotic muscle layer. The number of NGFR-stained epithelial cells in surface epithelium varied from numerous to abundant (Fig. 4) (Table).

In the proximal end wall, the number of NGFR-positive structures (basal epithelial cells and fine nerve fibers) was low (+) in 1 case, and moderate and numerous in 13 cases, while abundant number of positive cells was noticed only in 4 cases.

In the EA-affected tissues, moderate and numerous PGP 9.5-containing nerve fibers and bundles were detected around small blood vessels, within their walls, and inside the intermuscular nerve plexus (Fig. 5). The proximal esophagus showed a moderate number of PGP 9.5-containing nerves, while the nerves positive for this neuropeptide in the distal part of the esophagus were numerous. Within the esophageal epithelium, PGP 9.5-positive cells were not seen. In the control group, the numerous and abundant number of PGP 9.5-containing nerve fibers was observed (Fig. 6).

Comparing the numbers of structures expressing NGFR and neuronal structures containing PGP 9.5, a simultaneous increase in both of them was documented (Table). In the EA-affected cases, the abovementioned structures were mainly observed in moderate and numerous numbers. In cases of abundant PGP 9.5 expression, the number of NGFR-positive structures also increased.

In the control material, the number of TGF- β -positive structures varied from moderate to numerous (Fig. 7) in connective tissue and endothelium.

Commonly, TGF- β was found only in occasional cells of the EA-affected esophagus. Only in one case, it was observed in the moderate number of

endothelial cells (Fig. 8).

FGFR was found in abundance in both control and EA-affected specimens. FGFR was expressed by fibroblasts, macrophages, blood vessel endothelial cells, leukocytes, lymphocytes as well as nerve fibers. Comparison of FGFR expression with TGF- β one established a reciprocal dependence between these two factors, such as decrease of TGF- β -containing cell number coming along with enlarged FGFR expression.

A very weak VEGF expression in occasional endothelial cells was seen in both the control and EA-affected tissues only in 2 cases.

In the control material, EGFR-containing cells, such as fibroblasts and macrophages, were found in moderate numbers, while in the EA-affected tissues, EGFR was detected in small-to-moderate number of inflammatory cells within blood vessels only in a few patients, but in most cases, the appearance of EGFR was negative or insignificant in all other structures.

Fibroblasts, macrophages, endothelial cells, leukocytes, and lymphocytes were positive for MMP-2. In the control tissues, this metalloproteinase marked numerous structures. The MMP-2 expression was weaker in the proximal part of the esophagus in EA-affected tissues as compared with controls. In the meantime, EA-affected tissues of the distal esophageal part demonstrated MMP-2 expression even higher than in control material.

In the control material, structures positive for NGFRp75, PGP 9.5, TGF- β , FGFR, and MMP-2 were found to be numerous or abundant (Table). FGFR expression was similar in the distal and proximal parts of esophagus with EA as compared with control specimens. The number of PGP 9.5- and NGFRp75-containing neuronal structures was diminished in the proximal, but not in the distal part of the esophagus. Despite the fact that there were some variations in the expression of NGFR in the distal part of EA-affected esophagus, the appearance of the factor was similar to that in the control speci-

Table. Relative Occurrence of Growth Factors, Their Receptors, Innervation Markers, and Tissue-Degrading Enzymes in Esophageal Atresia-Affected and Control Specimens

Material	NGFRp75	PGP 9.5*	TGF- β *	FGFR	VEGF	EGFR*	MMP-2*
Proximal esophagus; n=2	+++	++*	0/+*	++++	0	0-0/+*	++*
Distal esophagus; n=13	++/++++	+++*	0/+*	++++	0	0-0/+*	+++/++++*
Control; n=12	+++/++++	+++/++++*	+++/++++*	++++	0	++*	+++*

Commonly, control specimens showed a similar relative occurrence of different factors from different parts of the esophagus, thus in the table shown as one value. *A statistically significant difference found between the groups by using of nonparametric statistics.

NGFRp75, nerve growth factor receptor p75; PGP 9.5, protein gene peptide 9.5; TGF- β , transforming growth factor beta; FGFR, fibroblast growth factor receptor; VEGF, vascular endothelial growth factor; EGFR, epidermal growth factor receptor; MMP-2, matrix metalloproteinase 2.

0, negative reaction; 0/+, occasionally marked structures in the view field; +, a few positive structures in the view field; ++, a moderate number of marked structures in the view field; +++, numerous marked structures in the view field; +++++, abundance of marked structures found in the view field.

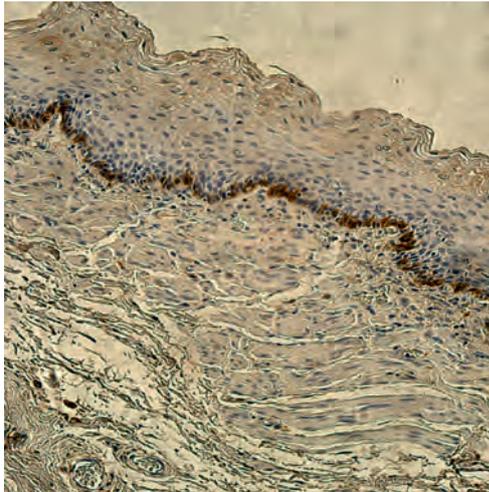


Fig. 1. Practically all basal epithelial cells show NGFR immunoreactivity in the control material (immunohistochemistry for NGFR, original magnification $\times 200$)

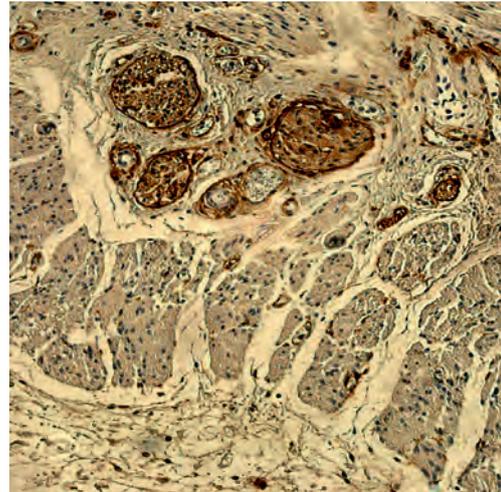


Fig. 2. NGFR-containing nerve fiber bundles and nerve fibers among smooth muscle cells in the wall of small blood vessels in the control material (immunohistochemistry for NGFR, original magnification $\times 250$)

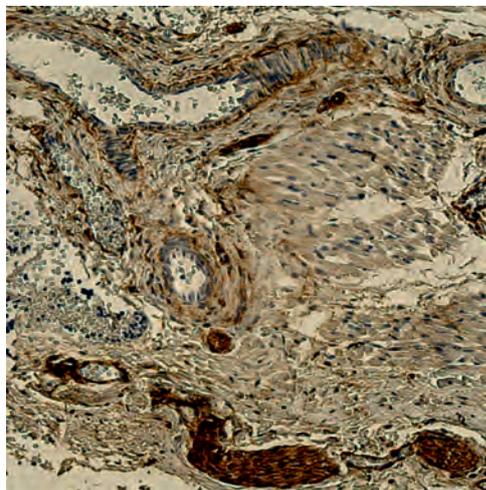


Fig. 3. NGFR-containing nerve fiber bundles and nerve fibers among smooth muscle cells in wall of small blood vessels in the esophageal atresia-affected material (immunohistochemistry for NGFR, original magnification $\times 250$)

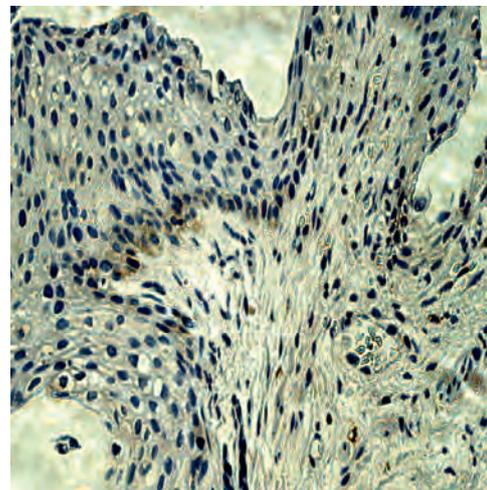


Fig. 4. Regional conglomerate of basal epithelial cells weakly stained for NGFR
Note also numerous vacuolized epithelial cells (immunohistochemistry for NGFR, original magnification $\times 400$)

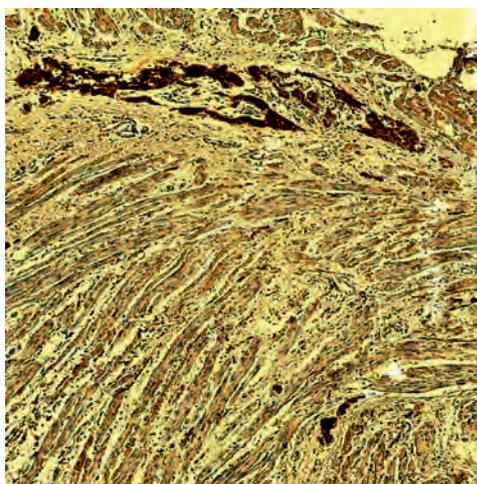


Fig. 5. PGP 9.5-containing nerve bundles in the intermuscular plexus and among smooth muscle in the esophageal atresia-affected specimen (immunohistochemistry for PGP 9.5, original magnification $\times 100$)

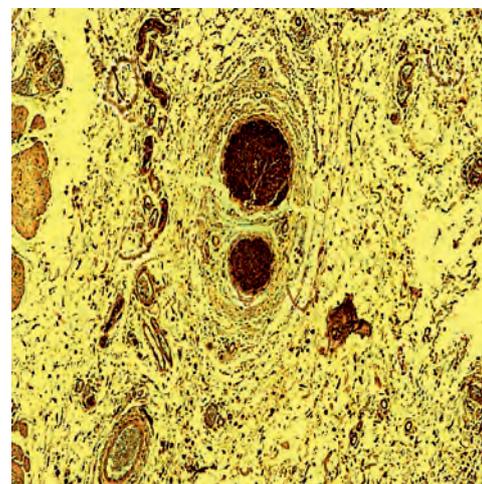


Fig. 6. PGP 9.5-containing nerve trunks and nerve fibers near the wall of small blood vessels in the control material (immunohistochemistry for PGP 9.5, original magnification $\times 100$)

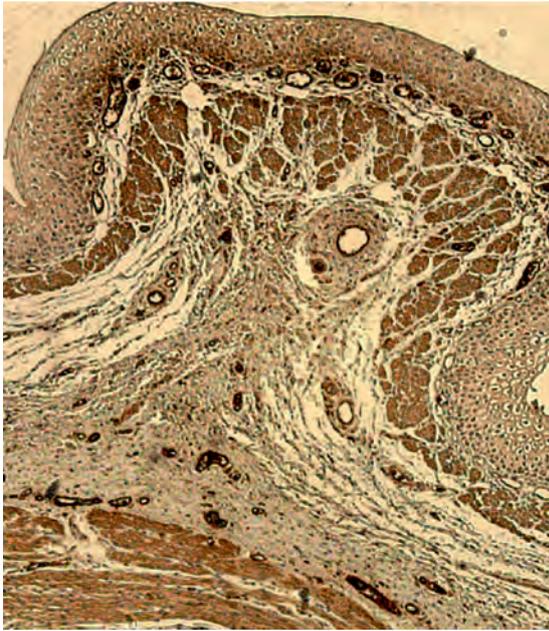


Fig. 7. TGF- β -containing endothelial cells in the control material (TGF- β IMH, original magnification $\times 100$)

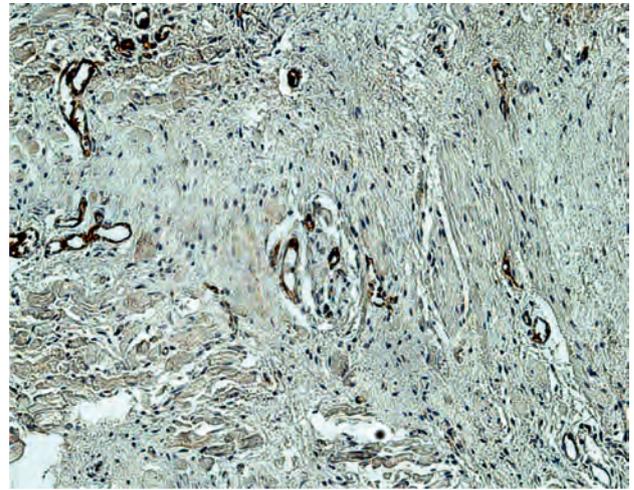


Fig. 8. TGF- β -containing endotheliocytes in the wall of blood vessels of the proximal esophageal pouch (TGF- β IMH, original magnification $\times 200$)

mens. The number of TGF- β -containing structures was notably decreased in the EA-affected tissue. EGFR expression was characteristic of control, but not of EA-affected tissue. There were no VEGF-positive structures in the esophagus of either control or EA-affected patients. The same or higher number of MMP-2-positive cells was seen in the specimens of the distal esophagus than in the control ones, while the specimens from the proximal part of the esophagus demonstrated a decreased number of MMP-2-positive cells.

Comparison of control and EA-affected specimens of the esophagus revealed a significant difference in the relative number of TGF- β -positive ($\chi^2=20.609$; $P=0.001$), EGFR-positive ($\chi^2=17.662$; $P=0.001$), and MMP-2-positive ($\chi^2=6.002$; $P=0.050$) structures between control and proximal/distal esophageal specimens. A significant difference in the structures positive for PGP 9.5 was observed between control/distal and proximal esophageal specimens ($\chi^2=7.847$; $P=0.020$) (Table).

Discussion

In the EA-affected tissues, the number of PGP 9.5-containing nerve fibers was lower compared with the control material. Generally, PGP 9.5 possesses intrinsic ligase and hydrolase activity necessary for a process of proteosomal protein degradation and supports the normal functioning of neuronal structures (23). Therefore, the decreased amount of PGP 9.5-positive structures might indicate a neuronal origin of the disorder. Interestingly, inherited innervation disorders are mentioned also in a study

by Qi et al. (1997), when branching abnormalities of the vagal nerve during embryogenesis result in nerve tissue deficiency particularly in the distal part of the esophagus. Such findings probably shed light to the abnormalities of esophageal motility established in patients with EA/TEF before operative treatment (24).

As compared with the control material, the number of NGFR-expressing structures was also diminished in the EA-affected proximal esophagus. However, there was no significant difference comparing the specimens obtained from both parts of EA-affected esophagus and control esophagus. According to the data reported by Bibel and Barde (25), NGFR binds not only NGF, but also other neurotrophins. Altogether, neurotrophins promote the formation of ceramides activating NGFR (26), but ceramides contribute to the protection of axonal structures and function. Thus, changes in NGFR expression might indicate damage to neuronal tissues.

In EA-affected tissues, TGF- β -containing endothelial cells were sparse. TGF- β is needed for the stabilization and further development of newly formed blood vessels (27). Diminished TGF- β expression very likely corresponds to cell proliferation, growth, and maturation abnormalities (14) as well as disorders of extracellular matrix formation. These disturbances, in turn, might lead to EA manifestation.

FGFR marked abundant structures in both control and EA material. Fibroblasts, macrophages, lymphocytes, blood vessel endothelial cells, leukocytes as well as nerve fibers expressed FGFR. In

other tissues of endodermal origin, for instance, healthy lung tissue, FGFR expression is spatially restricted. Although in case of mesenchyme contact with Shh negative epithelium during embryogenesis, there is also described rich FGFR expression in wide area that possibly causes branching disorders (28). The abovementioned mechanism could explain the origin of distal esophagus or fistular tract growing caudally as straight, nonbranched structure showing an abundant FGFR expression (29). Fusion between fistular tract and developing stomach could therefore compensate and/or restore continuity of the gastrointestinal tract (30).

VEGF expression was negative both in the control and EA-affected tissues, with only occasional positively stained endothelial cells in some cases. VEGF stimulates the proliferation of endothelial cells, particularly under hypoxia (16). A weak VEGF expression pattern is very likely explained by the most intensive angiogenesis in the course of embryogenesis, but our study material was obtained from newborns. It is interesting to note that VEGF possesses neurotrophic as well as neuroprotective properties (31). These VEGF properties and almost negative VEGF expression on the other hand might only partially explain the observed innervation disorders in EA-affected tissues, because the negative expression of growth factor was observed also in the control tissues. On esophageal occlusion, including also EA, enhanced VEGF expression is observed in amnion and chorion and promotes intramembrane fetal fluid absorption into fetal circulation as well as decreases the likelihood of occurrence of polyhydramnion (32).

EGFR-positive structures were found in moderate numbers in the control specimens, while EA-affected tissues displayed insignificant or even negative expression patterns. EGFR is found in the tissues of epithelial, mesenchymal, and neuronal origin, where it contributes to cell proliferation, differentiation, and development (17). Therefore, EGFR expression abnormalities, which differed sta-

tistically significantly between the patient and control groups, might be one of the mechanisms behind EA development.

In the control material, MMP-2 marked many structures so that in the proximal part of the esophagus, the number of positive structures was moderate, whereas in the distal part of esophagus, MMP-2-positive structures were numerous and abundant. MMP catalyzes extracellular matrix macromolecules, proteoglycans, and additional proteins such as fibronectin (33). According to the data of Okada et al. (2004), increased NGF expression comes along with increased MMP-2 expression (34). A decrease in NGFR-positive structures in our investigative material was often observed, but the difference between MMP-2 and NGFR relative occurrence was not proved to be significant. Nevertheless, in cases of denervation and diminished innervations, MMP-2 expression was not changed substantially. A study by Kherif et al. reported an increased number of MMP-2-positive cells observed in neuronal tissues with structural lesions indicating the role of MMP-2 in axonal degeneration and common regeneration (35).

Conclusions

A decrease in PGP 9.5-containing neuronal structures in the proximal esophagus supports insufficient innervation of this part of the organ in esophageal atresia. A decrease in MMP-2 positive cells in the esophageal atresia-affected proximal esophagus indicates also a possible decrease of tissue adaptive and regenerative reactions. Low expression of TGF- β and almost the absence of EGFR in the esophageal atresia-affected specimens may deal with disturbances in cell growth, proliferation, and differentiation, indicating a significant role of these substances in morphopathogenesis of esophageal atresia. FGFR and VEGF seem not to characterize esophageal atresia pathogenesis.

Statement of Conflict of Interest

The authors state no conflict of interest.

Vaiķu, sergančīu stemplēs atrezija, stemplēs proksimalinēs ir distalinēs daļīu augimo faktoriāi

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Raktažodžiai: augimo faktoriāi, imunohistochemija, stemplē, artrezija, vaikai.

Santrauka. *Įvadas.* Stemplēs atrezijos patogenezė iki šiol nepakankamai iširta, nors ši anomalija sąlyginai gausiai paplitusi populiacijoje: iš 3000 gyvū gimusiųjų vienas gimsta su stemplēs atrezija.

Tyrimų tikslas. Nustatyti augimo veiksnių ir jų receptorių neuropeptidų bei audinių irimo fermentų – matrikso metaloproteinazių – santykinį paplitimą (buvimą) stemplės proksimalinėje bei distalinėje dalyse esant stemplės atrezijai.

Medžiaga ir metodai. Histopatologiškai buvo iširta 15-kos pacientų stemplės atrezijos medžiaga. Panaudojant biotino–streptovidino imunohistocheminį metodą, nustatyti: nervų augimo faktorių receptoriai p75 (NGFR p75); proteino geno produktas 9,5 (PGP 9,5); transformuojantis augimo faktorius beta (TGF-β); fibroblastų augimo faktoriaus receptoriai 1 (FGFR1); kraujagyslių endotelio augimo faktoriai (VEGF); epidermio augimo faktorių receptoriai (EGFR); matrikso metaloproteinazės-2 (MMP-2).

Rezultatai. Kontrolinės grupės ir pacientų, turinčių atreziją, stemplės distalinėje dalyje NGFR p75 išraiška buvo gausi, tuo tarpu pacientų stemplės proksimalinėje dalyje nustatytas receptorių turinčių struktūrų skaičiaus sumažėjimas. Panašūs rezultatai gauti įvertinant PGP 9,5 teigiamas struktūras. Esant atrezijai stemplėje aptiktos tik pavienės teigiamos TGF-β ląstelės, o kontrolinės grupės tiriamųjų tyrimo medžiagoje tokių ląstelių buvo vidutiniškai daug ir labai daug. Tiek kontrolinės, tiek pacientų grupės tiriamųjų stemplėse buvo aptikta daug FGFR, tuo tarpu VEGF buvo tik pavienėse ląstelėse. Tik kontrolinėje grupėje buvo aptikta vidutiniškai daug jungiamojo audinio ląstelių su EGFR. Skirtingai nei kontrolinėje grupėje, esant atrezijai, MMP-2 teigiamų ląstelių skaičius stemplės proksimalinėje dalyje buvo sumažėjęs.

Išvados. Stemplės proksimalinėje dalyje sumažėjusių PGP 9,5 teigiamų nervinių stuktūrų kiekis patvirtina nepakankamą šios dalies inervaciją esant atrezijai. Sumažėjęs MMP-2 teigiamų ląstelių skaičius stemplės proksimalinėje dalyje rodo galimai mažėjantį audinių prisitaikymą ir regeneraciją. Esant atrezijai, silpna TGF-β raiška ir beveik visiškai EGFR nebuvimas gali sąlygoti ląstelių augimo, proliferacijos bei diferenciacijos pažaidas. Atrodo, kad FGFR bei VEGF atrezijos patogenezėje nebūdingi.

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