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Basic Fibroblast Growth Factor (bFGF), Fibroblast Growth Factor Receptor 1 (FGFR1), Transforming Growth Factor Beta (TGF-β) and Chromogranin A (CgA) Appearance in Congenital Intra-abdominal Adhesions in Children under One Year of Age

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Authors' contributions

This work was carried out in collaboration between all authors. Author AA managed the literature searches, performed the immunohistochemical and statistical analysis and wrote the first draft of the manuscript. Author MP designed the study and wrote the protocol. Authors ZA and OV managed the analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

Aim: Of this work was to determine dispersion of TGF-b, fibrosis modulating factor (bFGF and FGR1) and granule marker chromogranin A in case of intra-abdominal adhesion which could be essential factors in disease pathogenesis. Thus than could be used as possible biomarker.

Study Design: Observational study.

Place of Study: Institute of Anatomy and Anthropology and Department of Children Surgery, Rīga Stradiņš University.

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Materials and Methods: The specimens used for research were obtained from 50 patients aged 1 to 292 days. They underwent abdominal surgery due to obstructive gut malrotation and several additional pathologies. Tissues were processed for bFGF, FGFR1, TGF-β and CgA by means of biotin-streptavidin immunohistochemistry.

Results: In adhesion tissue bFGF positive connective tissue cells varied from a few to an abundant amount, but in 15 specimens no positive structure was observed. Few connective tissue fibers and moderate to numerous fibroblasts and macrophages contained FGFR1. A moderate number of TGF-β positive connective tissue fibers were observed. Some specimens also contained positive fibroblasts, macrophages and endotheliocytes. Few connective tissue cells contained CgA. A moderate correlation was observed between bFGF and FGFR1 (Spearman's rank correlation coefficient = .500, $P < .001$) as well as between bFGF and CgA (Spearman's rank correlation coefficient = .311, $P = .03$).

Conclusions: The connection between the less distinct bFGF and more prominent FGFR1 proves the compensatory stimulation of receptors as a response on the lack of the same factor in case of adhesion disease. Persisting appearance of TGF-β positive structures in congenital adhesions indicates the continuing growth/regeneration potential of loose connective tissue. Positive CgA structures indicate the involvement of the neuroendocrine system in case of adhesion disease.

Keywords: Children; adhesions; growth factors; Immunohistochemistry.

1. INTRODUCTION

Intestinal obstruction is one of the most common emergencies in pediatric surgery. Recent estimates place the incidence at 1 of 2000 live births [1]. Adhesive intestinal obstruction occurs, when pathologic bands are formed between the peritoneum, the small and large intestine, the abdominal wall and other intraabdominal organs [2]. Typically adhesions occur after intraabdominal surgery and peritonitis (regarded the most common complication after abdominal surgery [3,4], but rarely can also be congenital in their origin [5,6].

Congenital intra-abdominal adhesions occur due to disruption of the normal embryologic development [7]. During normal abdominal development, the 3 divisions of the GI tract (foregut, midgut, hindgut) herniate out from the abdominal cavity, where they then undergo a 270º counterclockwise rotation around the superior mesenteric vessels. Following this rotation, the bowels return to the abdominal cavity, with fixation of the duodenojejunal loop to the left of the midline and the cecum in the right lower quadrant [8,9]. Interruption of typical intestinal rotation and fixation during fetal development can occur in a wide range of locations [9].

As the embryonic development is tightly regulated by the complex interaction of several morphogenetic factors, which determine and modulate tissue growth. Degeneration, innervation, angiogenesis and fibrosis, fibrosis modulating factors may also play a role in the development of congenital intestinal adhesions. bFGF displays mitogenic, migratory, and morphogenic functions. It is also known to play a role in organ development, organ regeneration, and wound healing [10]. It can stimulate smooth muscle proliferation [11] and influence the adhesion characteristics of osteoblasts [12]. bFGF is a potent angiogenic factor and endothelial cell mitogen. Although bFGF levels are increased in chronically inflamed tissue, but its role in inflammation is unclear [13]. Increased levels of bFGF are found in body fluids of patients carrying large tumor burdens, which may allow the tumor to stimulate blood vessel ingrowth [14].

FGFs execute their biological actions by binding, dimerizing and activating cell surface FGF receptors [15]. bFGF activates FGFR1 in cooperation with heparin or heparin sulfate proteoglycan to induce its pleiotropic effects in different tissues and organs [16]. The FGF/FGFR system has been implicated in a variety of physiological and pathological conditions. This includes embryonic development, tissue growth and remodeling, inflammation, tumor growth, and vascularization. FGF/FGFR signaling plays important functions in mesoderm formation and development [17]. FGFR1 activation by bFGF promotes catabolism and impedes anabolism [18].

The results of several research works indicate the possible role of a increased TGF-β production in the etiology of adhesions [19,20].

TGF-β is a member of a family of dimeric polypeptide growth factors that includes bone morphogenic proteins and activins. TGF-β regulates the proliferation and differentiation of cells, embryonic development, wound healing, and angiogenesis. Increases or decreases in the production of TGF-β have been linked to numerous disease states, including atherosclerosis and fibrotic disease [21]. Increased TGF-β production is associated with adhesion development [22]. Fibroblasts obtained from adhesions produce greater amounts of TGF-β and extracellular matrix molecules than normal fibroblasts isolated from normal peritoneum [23]. TGF-β stimulates fibroblasts and other cells to produce extracellular-matrix proteins and cell-adhesion proteins (collagen, fibronectin, integrins). In the same time it decreases the production of enzymes that degrade the extracellular matrix (collagenase, heparinase, stromelysin). This increases the production of proteins that inhibit enzymes that degrade the extracellular matrix (plasminogen-activator inhibitor type 1, tissue inhibitor of metalloprotease) [21].

CgA is an acidic glycoprotein belonging to a family of regulated secretory proteins. It is stored in the dense core granules of the adrenal medulla and in many other neuroendocrine cells and neurons [24]. It is known that it acts as a precursor of many biologically active peptides generated by cleavage at specific sites [25]. It is the major soluble protein co-stored and co-released with catecholamines. CgA can function as a pro-hormone by giving rise to several bioactive peptides [26]. This protein is produced, in certain conditions also by cardiomyocytes, keratinocytes and granulocytes. Increased levels of circulating CgA have been detected in patients with cancer, heart failure, hypertension, atrophic gastritis, renal failure, giant cell artheritis, rheumatoid arthritis, sepsis and other inflammatory diseases [27].

Therefore, we suggest that bFGF, FGFR1, TGF-β and CgA could be of importance in the pathogenesis of adhesions and should be explored as possible biomarkers.

2. MATERIALS AND METHODS

The specimens used for research were obtained from 50 patients (23 males, 27 females) aged 1 to 292 days (Table 1) who underwent abdominal surgery. This was due to obstructive gut malrotation and several additional pathologies. These specimens are the property of the Institute for Anatomy and Anthropology of Rīga Stradiņš University.

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). These were then 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 μm in thickness by means of a microtome.

2.1 Immunohistochemical Analysis

Immunohistochemistry (IMH): bFGF (anti-bFGF, working dilution 1:200, Abcam, England), FGFR1 (anti-FGFR1, working dilution 1:100, Abcam, England), TGF-β (anti-TGF-β, working dilution 1:100, Santa Cruz, USA), CgA (anti-CgA, working dilution 1:100, Invitrogen, England) were used by biotin – streptavidin IMH [28].

Adhesion tissue tissue was deparaffinized and washed in alcohol and water, then washed for 10 minutes in wash buffer (Tris-buffered saline) and put for 5 minutes in EDTA boiling buffer in microwave. When the samples had cooled down, they were washed twice for 5 minutes in wash buffer. To decrease background staining normal blocking serum for 20 minutes was used. All tissue samples were incubated with primary antibodies for 1 hour. Further, washing for 10 minutes in wash buffer and incubation for 30 minutes with LSAB+LINK with biotin related secondary antibodies (code K1015, DakoCytomation, Denmark) was performed. Another washing for 5 minutes in wash buffer was performed. The specimens were incubated for 25 minutes with LSAB+LINK with enzyme peroxidase labeled streptavidine (code K0690, DakoCytomation, Denmark). It was followed by 5 minutes washing in wash buffer and 10 minutes processing with DAB substrate-chromogen system (code K3468, DakoCytomation, Denmark) to obtain positive structure staining in brown colour. Samples were then rinsed in running water and stained with hematoxylin.

Findings were evaluated using Leica DC 300F camera and image processing and analysis software Image Pro Plus.

Table 1. Patient information

	Gender	Age (days)	Location		Gender	Age (days)	Location		Gender	Age (days)	Location
1	M	0	SI	18	F	4	SI	35	M	56	SI
2	F	0	D	19	F	9	D	36	F	56	SI
3	F	0	LB	20	F	9	D	37	F	62	D
4	M	1	SI	21	F	9	AAW	38	M	67	SI
5	F	1	LB	22	M	9	LB	39	F	71	SI
6	M	1	SI	23	M	14	SI	40	F	94	SI
7	F	1	LB	24	M	15	LB	41	F	100	SI
8	F	1	D	25	F	15	LB	42	M	103	D
9	M	1	AAW	26	F	19	SI	43	M	108	DI
10	M	2	SI	27	F	26	LB	44	M	129	DI
11	M	2	SI	28	M	28	DI	45	F	130	D
12	M	2	D	29	F	30	SI	46	F	134	SI
13	M	2	LB	30	F	36	SI	47	F	151	SI
14	M	2	SI	31	M	39	D	48	F	185	SI
15	M	3	LB	32	F	41	LB	49	M	210	DI
16	M	4	D	33	M	48	LB	50	F	292	SI
17	F	4	LB	34	F	51	LB				

Abbreviations: SI – small intestine, DI – distal ileum, D – duodenum, LB – Ladd's band, AAW – anterior abdominal wall; m – male, f – female

For quantification of structures, the semiquantitative counting method was used. The designations were as follows:

- 0, no positive structures found in the visual field;
- 0/+, occasionally marked structures in the visual field;
- +, a few positive structures in the visual field;
- +/++ few to moderate positive structures in the visual field;
- ++, a moderate number of marked structures in the visual field;
- ++/+++, moderate to numerous number of positive structures in the visual field;
- +++, a numerous number of marked structures in the visual field;
- ++++, abundance of marked structures found in the visual field [29].

2.2 Data Analysis

For data storing and processing the Microsoft Office Excel 2010 was used. Data analysis was conducted using Statistical Package for the Social Sciences (SPSS) program version 20.0. Spearman's correlation test (rs) was used to evaluate correlation in between growth factors. Correlation was considered as weak if value rs was 0 – 0.3, moderate, if value rs was 0.31 – 0.69 and strong if value rs was 0.7 – 1. Two-tailed *P* values of <.05 were considered as statistically significant.

3. RESULTS

bFGF was seen exclusively in fibroblasts and macrophages (Fig. 1A). In four cases the number of marked cells was abundant (++++), in five cases numerous (+++), but in two cases moderate to numerous (++/+++). Eleven specimens showed a moderate (++) number of bFGF positive cells. Two specimens showed few to moderate (+/++) bFGF positive cells, seven – few (+) positive cells. A positive reaction to bFGF was rarely seen for endotheliocytes and connective tissue fibers. Occasional (0/+) positive fibroblasts and macrophages were observed in four cases. 15 specimens didn't contain any bFGF containing structures (Table 2).

A positive reaction for FGFR1 was observed in fibroblasts, macrophages and connective tissue fibers. Rarely seen were FGFR1 positive endotheliocytes. A numerous (+++) number of FGFR1 positive fibroblasts and macrophages were observed in five cases (Fig. 1B). In another

eight cases a moderate to numerous (++/+++) number of positive structures was marked. FGFR1 positive structures were mostly seen in moderate (++) and few to moderate (+/++) appearance. Few (+) fibroblasts and macrophages contained this factor in nine specimens. Occasional (0/+) positive structures were observed in three cases (Table 2).

A numerous (+++) number of TGF-β positive structures was found in five specimens (Fig. 1C), but a moderate to numerous (++/+++) number was observed in 14 specimens. A moderate (++) number of TGF-β positive fibers was observed in 20 cases, in another eight cases few (+) numbers of these structures were positive for TGF-β (Table 2).

In two cases the number of CgA containing structures was numerous (+++), in another two cases moderate to numerous (++/+++), but in five cases moderate (++) (Fig. 1D and 1E). Seven specimens showed few to moderate (+/++) and 14 specimens few (+) numbers of CgA positive structures. In 18 specimens no positive structures were observed (Table 2).

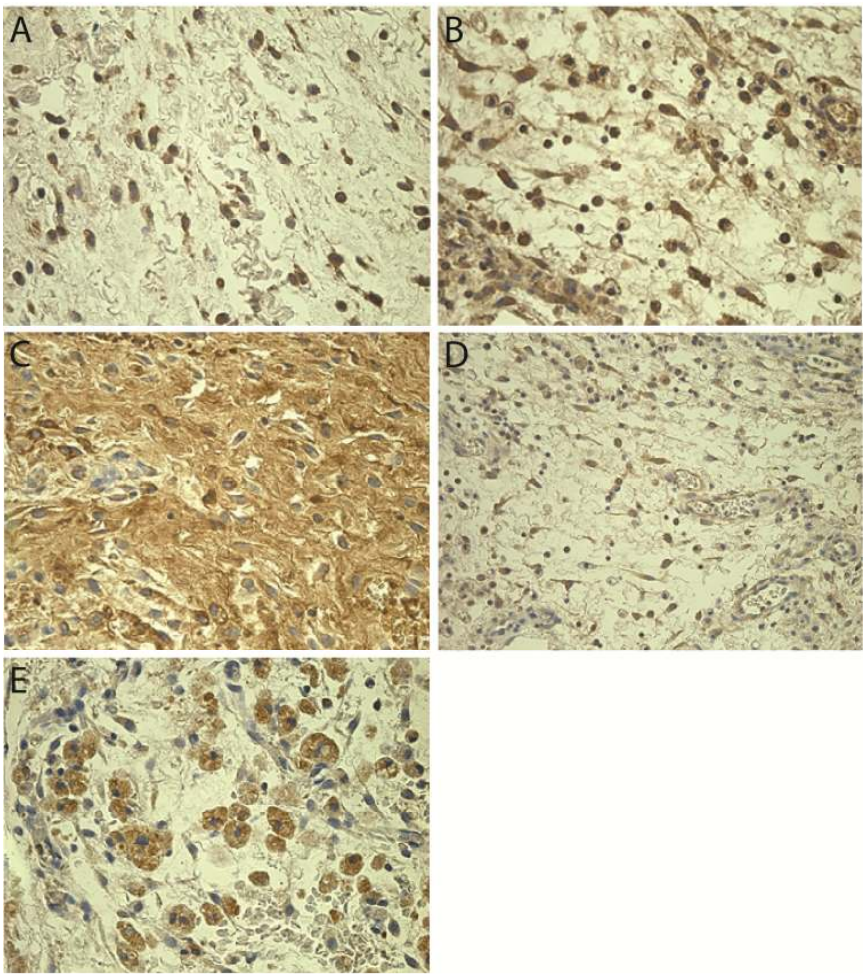

Fig. 1. Immunoreactive structures in congenital adhesions

A Moderate bFGF positive fibroblasts and macrophages in congenital adhesions of the four days old patient (bFGF IMH, x400); B Numerous FGFR1 positive fibroblasts and macrophages in congenital adhesions of the two days old patient (FGFR1 IMH, x400); C Numerous TGF-β positive fibroblasts, macrophages and fibers in congenital adhesions of a three months old patient (TGF-β IMH, x400)
D Moderate CgA positive fibroblasts and macrophages in congenital adhesions of a four days old patient (CgA IMH, x250); E Moderate CgA positive epithelioid cells in congenital adhesions of a two days old patient (CgA IMH, x400)

Table 2. Semi-quantitative evaluation of immunoreactive structures

	bFGF	FGFR1	TGF-β	CgA
0	15	0	0	18
0/+	4	3	0	2
+	7	9	3	14
+/++	2	12	8	7
++	11	13	20	5
++/+++	2	8	14	2
+++	5	5	5	2
++++	4	0	0	0

Abbreviations: bFGF – basic fibroblast growth factor, FGFR1 – fibroblast growth factor receptor 1, TGF-β – transforming growth factor β, CgA – chromogranin A, 0 – no positive structures, 0/+ – occasionally positive structures, + – a few positive structures, +/++ – few to moderate positive structures, ++ – a moderate positive structures, ++/+++ – moderate to numerous positive structures, +++ – a numerous positive structures, ++++ - abundant positive structures.

Using the Spearman's correlation test positive correlations were observed between the immunoreactive structures for bFGF and FGFR1 ($rs = .500$; $P < .001$) and immunoreactive structures for bFGF and CgA ($rs = .311$; $P = .03$).

4. DISCUSSION

TGF- β is the most studied cytokine in the pathophysiology of adhesion development, and has been suggested as principal profibrotic mediator of this process [30]. TGF-β is a key regulator of extracellular matrix assembly and remodeling [30,31]. The action of TGF-β following inflammatory responses is characterized by increased production of extracellular matrix components, as well as mesenchymal cell proliferation, migration, and accumulation [32]. TGF-β causes matrix deposition in mesenchymal cell in culture by promoting expression of extracellular matrix genes. These suppress the activity of genes such as matrix metalloproteinases, which degrade extracellular matrix [33,30]. In our study TGF-β was seen in moderate to numerous numbers in almost all analyzed specimens. This is in agreement with previous studies of TGF- β in adhesions. The overexpression of TGF- β has been linked to tissue fibrosis at various sites throughout the body including peritoneal adhesion formation [34]. Elevated TGF-β expression in affected organs, and subsequent deregulation of TGF-β functions, correlates with an abnormal connective tissue deposition [30]. It has been reported, that suppression of the actions of TGF- β reduced adhesion formation [35] and anti-TGF-β agents were effective against postoperative inflammation and fibrosis [36].

The pathogenesis of congenital intra-abdominal adhesions could possibly be related to bFGF/FGFR1 signaling modification, which in turn could be responsible for inflammation and changes in vascularization and tissue development, as well as growth and remodeling processes. In one third of the analyzed specimens numbers of bFGF positive structures were low or no positive structures were detected. There are publications describing a possible loss of bFGF from the cell cytoplasm through plasma membrane disruptions caused by physiologically generated mechanical force [37]. In contrast to that, almost in a half of the analyzed specimens bFGF positive structures were observed at least in moderate counts and more. Studies show that elevated levels of bFGF are implicated in the pathogenesis of several diseases characterized by a disregulated angiogenic/inflammatory response. bFGF induces the expression of a inflammation-related genes in endothelial cells, including pro-inflammatory cytokines/chemokines and their receptors, endothelial cell adhesion molecules, and components of the prostaglandin pathway [38].

To our knowledge no studies have looked at the presence of FGFR1 in adhesions. We found moderate to numerous FGFR1 containing cells in intra-abdominal adhesions. Elevated expression of FGFR1 is believed to disrupt the interplay between mesenchymal and epithelial cells [39]. FGF/FGFR signaling plays important functions in the mesoderm [17]. In our study a significant correlation between bFGF and FGFR1 was detected.

It is also possible that the neuroendocrine system is involved in the pathogenesis of congenital adhesions. Positive structures for CgA were seen in few to moderate numbers in the analyzed specimens. CgA is concentrated and stored within granules and rapidly released by neuroendocrine cells and neurons after an appropriate stimulus, this protein could be important for the local control of cell adhesion by stimulated cells [40]. CgA, locally administered to injured mice, can accelerate keratinocyte proliferation and wound healing, regulate keratinocyte adhesion and migration [41].

5. CONCLUSIONS

Connection between the less distinct bFGF and more prominent FGFR1 proves the compensatory stimulation of receptors as a response on the lack of the same factor in case of adhesion disease.

Persisting appearance of TGF-β positive structures in congenital adhesions indicates the continuing growth/regeneration potential of loose connective tissue.

Positive CgA structures indicate the involvement of the neuroendocrine system in case of adhesion disease.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

The study was approved by the Ethical Committee at Rīga Stradiņš University, permit issued on May 10, 2007.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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