Increase in pro-apoptotic Bax expression and decrease in anti-apoptotic Bcl-2 expression in newborns with necrotizing enterocolitis

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ABSTRACT

Background/Aim. The aim of the present study was to find out if there is an increase in the expression of pro-apoptotic Bax and reduction in expression of anti-apoptotic Blc-2A1 in newborn intestines with necrotizing enterocolitis (NEC).

Material and Methods. We compared 8 consecutive newborn patients undergoing bowel resection for NEC with 8 neonates undergoing intestinal resection for ileal atresia.

Histopathological evaluation of tissue injury and apoptosis was performed by using light microscopic examination and TUNEL method. The mRNA level of apoptotic (CASP3, CASP6, CASP7, Bax, BIRC2) and anti-apoptotic genes were evaluated by PCR array method. Protein expression was assessed by immunohistochemistry.

Results. Tissue injury scores and mean apoptosis scores were significantly higher in NEC group when compared with control group ($p < 0.01$). Expression of pro-apoptotic genes were significantly increased in NEC group when compared with control group ($p < 0.01$). Expression of anti-apoptotic Bcl-2A1 gene was significantly decreased in NEC group, ($p < 0.01$). Protein expression of Bax and CASP3 was significantly increased in NEC group, ($p < 0.01$).

Conclusion. Our data in human newborns suggest that alteration of the balance between pro-apoptotic Bax expression and anti-apoptotic Bcl-2A1 expression in the site of injury is a possible mechanism in the pathogenesis of NEC.

Keywords: *apoptosis, gene expression, necrotizing enterocolitis, newborn.*

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INTRODUCTION

Necrotizing enterocolitis (NEC) is a complicated, multi-factorial condition of newborns and premature infants which causes significant mortality and morbidity in this population. NEC is characterized by intestinal epithelial cell apoptosis, necrosis, haemorrhage, incomplete enterocyte migration, and proliferation that results in persistent gut barrier failure which leads to loss of epithelial integrity, invasion of the intestine by bacteria followed by an acute, hyper-reactive inflammatory reaction and consecutive bowel necrosis.[1,2]

The balance between cell proliferation and cell loss is necessary for the maintenance of intestinal epithelial homeostasis.[3,4] The majority of cell loss in the normal intestine occurs by apoptosis.[3] The balance of pro-apoptotic and anti-apoptotic proteins is crucial for cell survival.[3] Bcl-2 family is a significant class of molecules that control enterocyte apoptosis.[3] Bcl-2A1 is an anti-apoptotic protein which inhibits the cytochrome *c* release from the mitochondria and reverses the effects of the pro-apoptotic protein Bax.[3] It has been showed that an exaggerated epithelial apoptosis in gut leads to severe NEC injury.[2,3,5] In a rat model of NEC, it has been shown that expression of apoptotic genes increased whereas expression of anti-apoptotic genes decreased. Furthermore, prevention and reducing apoptosis in experimental settings have been suggested to reduce NEC incidence.[5-7] Therefore, we aimed to find out if there is an increase in the expression of pro-apoptotic genes and reduction in expression of anti-apoptotic genes in newborn intestine with severe NEC and compared with intestines of newborns with ileal atresia. We especially focused on expression of pro-apoptotic Bax and caspase-3 and anti-apoptotic Bcl -2A1 genes.

MATERIAL AND METHODS

The Ethics Committee at Ankara University, School of Medicine, approved the collection of operative specimens for experimental purposes (Protocol No: 13-271). The study was carried

out in accordance with the Helsinki Declaration. Informed consents were obtained from the parents. We compared 8 newborn patients undergoing bowel resection for NEC with 8 neonates undergoing intestinal resection for ileal atresia. Presence of free air and portal venous gas in direct radiograms, clinical deterioration while receiving optimal medical management were the criteria for surgical exploration in newborns with NEC. Diagnosis of NEC was confirmed during routine pathologic investigation. At laparotomy, 1x1 cm ileal segments of the surgical specimens were obtained and divided into two pieces. One of them was snap-frozen in liquid nitrogen at -80 °C for polymerase chain reaction (PCR) studies and the other was reserved in formaldehyde for histopathological examination. Histopathological evaluation of tissue injury was performed by using light microscopic examination and tissue injury grading score. Histopathological evaluation of apoptosis was performed by terminal deoxynucleotidyl transferase mediated dUPT-FITC nick end labeling (TUNEL) method.[2,8] The messenger ribonucleic acid (mRNA) levels of apoptotic and anti-apoptotic genes were evaluated by PCR Array method.[9] Protein expression of these genes was assessed by immunohistochemistry.[10]

TABLE 1: *Clinical characteristics, tissue injury and apoptosis scores of the patients in necrotizing enterocolitis (NEC) group*

NEC group Patients	Gender	Gestational Age (weeks)	Age at operation (days)	Weight (gram)	Extent of bowel compromise at operation	Operative procedure	Tissue injury grading system score	Apoptosis score
1	Male	30	20	1250	15 cm bowel necrosis	Ileal resection and ileostomy	3	3
2	Female	25	7	760	25 cm bowel necrosis and perforation	Ileocolic resection and ileostomy	4	4
3	Female	32	15	1530	30 cm bowel necrosis and perforation	Ileocolic resection and ileostomy	4	4
4	Male	27	17	850	20 cm bowel necrosis and perforation	Ileocolic resection and ileostomy	3	4
5	Male	33	11	1600	35 cm bowel necrosis and perforation	Ileocolic resection and ileostomy	4	4
6	Male	31	10	1420	10 cm bowel necrosis	Ileal resection and ileostomy	2	3
7	Female	29	8	1050	15 cm bowel necrosis and perforation	Ileocolic resection and ileostomy	3	3
8	Male	28	12	930	15 cm bowel necrosis and perforation	Ileal resection and ileostomy	2	3

cm: centimeter

TABLE 2: *Clinical characteristics, tissue injury and apoptosis scores of the patients in ileal atresia group*

Ileal Atresia Group Patients	Gender	Gestational Age (weeks)	Age at operation (days)	Weight (gram)	Findings at operation and operative procedure	Tissue injury grading system score	Apoptosis score
1	Female	32	3	1600	Type 3A ileal atresia ileoileal anastomosis	2	2
2	Male	36	2	2200	Type 2 ileal atresia ileoileal anastomosis	1	1
3	Male	38	2	3100	Type 2 ileal atresia ileoileal anastomosis	1	1
4	Female	33	4	1540	multiple ileal atresia Two ileoileal anastomosis	3	3
5	Female	40	2	3300	Type 2 ileal atresia ileoileal anastomosis	1	1
6	Male	38	2	2700	Type 3A ileal atresia ileoileal anastomosis	1	2
7	Female	37	2	2750	Type 1 ileal atresia ileoileal anastomosis	1	1
8	Male	38	2	2930	Type 2 ileal atresia ileoileal anastomosis	1	1

Statistical analysis

Apoptotic score and tissue injury grades were statistically evaluated by using the Mann-Whitney U test. Gene transcription studies and protein expressions were evaluated by Student's t-test. Statistical significance was considered as $p < 0.01$.

RESULTS

The clinical characteristics of the control (ileal atresia) and NEC groups are presented in *Tables 1 and 2*. The control patients had a significantly higher weight than the NEC cases. All NEC patients were premature and their mean gestational age at birth was 29.3 ± 2.6 weeks and their mean weight at surgery time was 1171 ± 321g. Median age of NEC group was 12.5 days (7-20) and median age of control group was 2.3 days.[2-4] Two of the eight control patients were premature infants. Control patients mean gestational age at birth was 36.5 ± 2.7 weeks and their mean weight at surgery time was 2515 ± 666 g. Indications for surgery in NEC group included pneumoperitoneum (*n*= 5) and failure of medical management associated with persistent dilated loop on x-ray (*n*= 2) or portal venous air (*n*= 1). All NEC cases had significant bowel necrosis which required bowel resection and ileostomy at surgery. In all control patients ileal atresia was corrected by ileoileal anastomosis.

Light microscopic evaluation of the samples stained with H&E revealed minimal separation of superficialepithelial cells from lamina propria in 6 patients with ileal atresia. Submucosal separation in one patient and both submucosal and lamina propria separation in the remaining patient was encountered. The patient who had both submucosal and lamina propria separation was a premature infant with type IV atresia. Severe separation of submucosa and lamina propia, edema in submucosa and muscular layer and loss of epithelial integrity was present in 4 patients with NEC. Transmural necrosis was evident in the other 4 patients with NEC. Tissue injury grading scores according to groups are shown in *Table 3*. Tissue injury scores were significantly higher in NEC group when compared with control group (3.3 ± 0.8 versus 1.5 ± 0.8, $p < 0.01$).

Mild apoptosis in the superficial epithelium and apoptosis including villi and crypts was encountered in samples from 7 and 1 patients with ileal atresia, respectively. All samples from the patients with NEC revealed either apoptosis including villi and crypts (n: 4) or transmural apoptosis(n: 4). The results of apoptosis scores according to groups are shown in *Table 4*. The mean apoptosis scores were significantly higher in the NEC group when compared with those in control group, (3.5 ± 0.5 versus 1.6 ± 0.5, $p < 0.01$).

The difference in the expression of apoptotic and anti-apoptotic genes between groups are shown in *Table 5*. The mRNA level of pro-apoptotic genes which are caspase-3, caspase-6, caspase-7, BIRC2 and Bax genes were significantly increased in NEC group when compared with control group($p < 0.01$). mRNA levels of anti-apoptotic Bcl-2A1 gene were significantly decreased in NEC group when compared with control group ($p < 0.01$). However, there was no statistically significant difference in mRNA levels of caspase-1, caspase-2, caspase-9 Bad, Bcl-X_L and Bcl-w genes between two groups.

Protein expression values are shown in *Table 6*. Mean protein expression percentage of Bax and Caspase 3 was significantly elevated in NEC group when compared withileal atresia group.

TABLE 3: *Tissue injury grades according to groups*

Groups	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
Necrotizing Enterocolitis Group (n= 8)	-	-	2	3	3
Control Group (n= 8)	-	6	1	1	-

TABLE 4: *Apoptosis scores according to groups*

Groups	Score 0	Score 1	Score 2	Score 3	Score 4
Necrotizing Enterocolitis Group (n= 8)	-	-	-	4	4
Control Group (n= 8)	-	5	2	1	-

DISCUSSION

This study showed that expression of pro-apoptotic Bax increased whereas expression of anti-apoptotic Bcl-2 genes decreased. In another study ileal samples obtained from infants undergoing surgical resection for NEC demonstrated higher levels of BNIP3 protein and authors proposed that upregulation of the cell death-related protein BNIP3 is a possible mechanism associated with enterocyte death observed in NEC.[6] More recently, the sentinel immune receptor Toll-like receptor 4 (TLR4) has been identified and activation of this receptor by bacterial lipopolysaccharide has been proposed to be directly responsible for increased rates of apoptosis and impaired mucosal healing.[11,12] An experimental study on mice with deficient TLR4 signaling showed that LPS-induced intestinal apoptosis was prevented and the incidence of NEC development reduced.[11] Furthermore, glutamine has been shown to downregulate Toll-like receptor 2 (TLR-2) and TLR-4 expression and to protect intestine in premature neonatal rats with NEC.[12] These studies provide a rationale for studying how the regulation of enterocyte apoptosis occurs within the human newborn intestine during development of NEC.

In this study, we investigated the expression of pro-apoptotic and anti-apoptotic genes, in intestines of human newborns with acute NEC. Consistent with previous reports, we detected high grade tissue injury and extensive apoptosis in enterocytes of infants with NEC when compared to controls with intestinal atresia.[2,6,7,13] There were some degree of mild tissue injury and apoptosis in the samples from ileal atresia patients who served as our controls. It could be argued that intestinal atresia patients might not have normal tissues. We took the samples from the grossly viable looking bowels which we expected to function normally. The mild changes observed in ileal atresia patients could be reflection of the mucosal injury which might be the result of prolonged intestinal dilatation or intestinal manipulation during surgery. The increased tissue injury and apoptosis scores encountered in one of the patients could either directly related to type 4 intestinal atresia which is more severe type than the others or delayed surgery which was done postnatal day 4 in this patient.

We detected increased expression of apoptotic caspase-3, caspase-6, caspase-7, BIRC2, and Bax genes and decreased expression of anti-apoptotic

TABLE 5: *The change in the expression of apoptotic and anti-apoptotic genes*

Genes	Function	The change in the expression of genes
Caspase-1	Apoptotic, cysteine peptidase	+ 0.68 ± 0.10
Caspase-2	Apoptotic, cysteine peptidase	+ 1.15 ± 0.07
Caspase-3	Apoptotic, cysteine peptidase	+ 6.70 ± 1.06*
Caspase-6	Apoptotic, cysteine peptidase	+ 5.33 ± 1.60*
Caspase-7	Apoptotic, cysteine peptidase	+ 10.52 ± 2.12*
Caspase-9	Apoptotic, cysteine peptidase	+ 1.21 ± 0.02
BCL2A1	Bcl-2 related protein A1, Anti-apoptotic	- 5.42 ± 1.32*
BIRC2	Baculoviral IAP repeat containig 2, apoptotic	+ 5.20 ± 1.16*
BAX	Bcl-2 Associated X protein, apoptotic	+ 27.24± 5.16*

n= 8, Mean ± SDV, *p <0.01

TABLE 6: *Caspase 3 and Bax protein expression in the intestinal tissue*

	Control group			Necrotizing enterocolitis		
	Patologist 1	**Patologist 2**	***Avarage**	**Patologist 1**	**Patologist 2**	***Avarage**
Caspase 3 expression	34.33 ± 4.73	32.67 ± 4.16	33.5 ± 4.44	93.33 ± 5.69	86.67 ± 7.57	**90.00 ± 6.63
Bax expression	26.02 ± 3.53	29.42 ± 3.24	27.72 ± 3.39	95.33 ± 6.96	98.71 ± 8.69	**97.02 ± 7.66

* % expression **P <0.01

Bcl-2A1 gene in tissue samples of neonates with NEC. Mean protein expression percentage of Bax and caspase-3 was significantly elevated in NEC group when compared with ileal atresia group. There were no statistically significant differences in Bad, Bcl-xL, and Bcl-w expression between NEC and ileal atresia intestines in our study. These findings of our study are in agreement with the previous experimental neonatal NEC study.[5] Bcl-2 and Bax proteins together compose a cellular rheostat which regulates cellular transition towards or away from apoptosis.[3,14] Our data showing increased caspase-3,caspase-6 and caspase-7 gene and caspase-3 protein expression collaborate with the findings of previous study which shows supplementation with a pan-caspase inhibitor normalized the rates of apoptosis leading to decreased NEC incidence in a neonatal rat model of NEC.[2]

Treatment modalities which could modulate the ratio of Bax to Bcl-2 proteins in favor of intestinal cell survival can prevent progression of apoptosis and development of NEC. Epidermal Growth factor (EGF) is one of the active growth factors which is naturally found in human milk. Clark et al. showed that in newborn rats with NEC supplementation of EGF in formula markedly decreased the Bax to Bcl-2 mRNA and protein ratios and dramatically reduced apoptosis.[5,15] Preliminary clinical trials using intravenous recombinant EGF in neonates diagnosed with NEC have been shown to increase repair of intestinal epithelium by inducing crypt cell proliferative activity.[15] The beneficial effects of EGF supplementation in neonates might depend on promotion of healing of intestinal mucosal barrier, therefore prevention of development of NEC.

CONCLUSION

Our data in human newborns suggest that alteration of the balance between pro-apoptotic Bax expression and anti-apoptotic Bcl-2A1 expression can be a possible mechanism in the pathogenesis of NEC. Our study provides basis to further studies to understand the mechanisms that lead to alteration of the balance between pro-apoptotic and anti-apoptotic gene expression and to design future therapeutic and/or preventative strategies for NEC by modifying apoptosis. ■

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