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The significance of fas/fasl expression in celiac disease, non-specific duedonitis and in duedonum biopsies showing increase in intraepithelial lymphocytes

Nurdan Tatar[1], Serdar Yanık[1], Ayse Neslin Akkoca[2, *], Zeynep Tugba Ozdemir[3], Mustafa Calıskan[4], Didem Sozutek[5], Pınar Atasoy[6]

[1]Iskenderun State Hospital, Department of Pathology, Hatay, Turkey
[2]Iskenderun State Hospital ,Department of Family Medicine, Hatay, Turkey
[3]Bozok University Faculty of Medicine, Department of Internal Medicine, Yozgat, Turkey
[4]Ankara Dışkapı Yıldırım Beyazıt Research Hospital, Department of Endocrinology, Ankara, Turkey
[5]Iskenderun State Hospital, Department of Internal Medicine, Hatay, Turkey
[6]Kırıkkale University Faculty of Medicine, Department of Pathology, Kırıkkale, Turkey

Email address:
ayseneslinoguzhan@hotmail.com (A. N. Akkoca)

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Abstract: Aim: Celiac disease (CD) is a chronic malabsorbtion disease of the small bowel. With a prevelance of about %1 it is a common disease in the community. FAS-FASL system which induces apopitosis is one of the most important pathways and responsible fort he development of mucosal atrophy in CD. The aim of this study is to investigate the patients who has the increase of intraepithelial lymphocytes (İEL) in duedonal mucosa and non specific duedonitis (NSD) and significance of FAS-FASL expression in these patients to distinct from CD.Materials and methods: 29 adults and 7 children celiac patients (with a preliminary diagnosis as iron deficiency anemia) and 17 adults and 6 children non-specific duedonitis patients included in the study. CD3, CD8, FAS and FASL expression were examined immunohistochemically from sections prepared from paraffin blocks in 28 adults and 7 children with normal duedonal mucosa and 24 adults and 6 children who has the increase of İEL in duedonum.Results: İn all groups; the number of İEL seen much more with CD3 in HE (hematoxylin eosin) sections . Again in all groups it is determined that majority of İEL expresses CD3 and CD8. Both in adult and children group; in surface and crypt enterosits in İEL ; the highest FAS expression was seen in enterosits and lamina propria cells in CD .More expression was determined in patients with the group who has İEL in duedonum and with NSD when compared to control group. FASL expression is increased in CD in comparison with normal but it was low in the group who has İEL in duedonum and NSD group. We found that FAS-FASL expression is not only an effective mechanism in pathogenesis of CD. We suggest that the functional significance of FAS expression should be investigated by methods of evaluating apopitosis in patients with İELAG and NSD who has more FAS expression when compared to CD . Also in the detection of number of İEL in suspected cases routine use of CD3 immunohistochemical evaluation may be useful.

Keywords: Celiac Disease (CD), İncreased İntraepithelial Lymphocytes (İEL),Non-Spesific Duedonitis(NSD), FAS, FASL

1. Introductıon

Celiac disease (CD) (Gluten-sensitive enteropathy) is a malabsorption disease charactarized with damage in small bowel mucosa which caused by the intake of gluten with diet in genetically suspectible individuals. İt is the most common cause of malabsorption in Eastern societes (1). İt is clinically important because it has %1 of the frequent occurence in society (2)and if not treated there is a risk of developing lymphoma and carcinoma (3).

CD diagnosis with clinical findings, serological tests and small bowel biopsy. İt may exhibit varying degrees of histological appearance from only the increase in

intraepithelial lymphocytes(İEL) in normal mucosa entirely up to the loss of absorptive villis structure.These changes may ocur in disorders other than CD so that clinical findings should be diagnosed in the presence of (4) .

Apoptosis is involved in the regulation of tissue hemostasis and is the the mechanism of cell death which is genetically controlled(5). Apoptosis provides construction-demolition of normal enterocytes and this assume the role of keeping epithelial function of the gastrointestinal tract(6). İt is thought that one of the most important mechanism in the formation of mucosal damage in CD is increasing enterocyte apoptosis(7) that is triggered by FAS-FASL interactions (8).There are many reasons to suggest that FAS-FASL pathway is of particular importance in diseases of the gastrointestinal tract. Most cells in the gastrointestinal tract expresses FAS and they were found to show high sensitivity to FAS-mediated death(9).

FAS-FASL apoptotic pathway was found to be effective in inflammatory processes especially in CD (10,11,12) and in Graft Versus Host Disease(GVHD) (13), Inflammatory BowelDisease (IBD) (14,15) and Helicobacterpylori infection(16,17,18). Immune-mediated diseases, such as in CD increased number of enterocytes along crypt-villus axis goes to premature apoptosis (19).

İn CD,cause of mucosal flattening is thought to be the perforin lytic granules which injures enterocytes and FAS / FASL-mediated increased enterocyte apoptosis (19,20). Small intestine IEL consist of T lymphocytes heterogeneous populations. Normally most of İEL are CD3 + CD8 +T cells and are mostly TCR-αβ + cells, CD4 +İEL creates very small portion. TCR-γδ expressing CD3 + CD8 cells are increased in untreated celiac patients. Increasing of IEL in CD is not specific therefore immunophenotyping of İEL may benefit to show the superiority of CD3 +CD8- cell (21).

İn this study importance of FAS and FASL expression was investigated in unknown etiology of duodenal mucosa which has increase in İEL and in patients with non-specific duodenitis (NSD) to distinction from CD.

2. Materials and Methods

Case Selection: 29 adult and 7 children were diagnosed as CD, 17 adult and 6 children were diagnosed as NSD, 24 adult and 6 children were diagnosed as the group who has İEL in duedonum. 28 adults and 7 children who has normal duodenal mucosa biopsies were used as controls. All of the patients has endoscopic biopsies taken from the second part of the duodenum. HE stained slides of the cases re-evaluated to assess to select villus orientation appropriate cases.

Histopathologic Review: Patients with CD were evaluated according to the criteria of Modified Marsh; in adult group 20 of them with Type IIIc, 8 patients with Type IIIb and 1 patient with Type IIIa; in children group 5 patients with Type IIIc and 2 patients were defined as Type IIIb as destructive type. Up to the Modified Marsh criteria; the group who has İEL in duedonum were not compatible in morphological features when compared with CD . When assessing the increase rate of İEL we considered normal rate as 100 enterocytes to 20 lymphocytes(101). Cases with NSD were ulcer-free patients, the specific etiology can not be determined and they were also showing increase in chronic inflammatory cells in the lamina propriain biopsies .

Immunohistochemistry: CD3 antibody as the primary antibody (Neomarkers, Ltd.), CD8 antibody (Neomarkers, Ltd.) and FAS antibody (Rabbit Pab, LabvisioLtd.) was used. FAS antibodies were prepared in optimal dilution in a portion of 1/100 .

For immunohistochemical analysis, the following operations were performed:

1. 4 μm thickening sections taken to adhesive blades and were left in the oven overnight at 37° C
2. The following day left in the oven at 60 ° C for 20 minutes and they were deparaffinized by xylene twice at 10 minute period with and passed from alcohols two times 2 minutes.
3. Sections bathe with distilled water taken to citrate solution (pH = 6) for antigen reveal phase and were incubated at 98 ° C for 23 minutes in PT modulation (Labvisio, UK) . and they were placed to automatic system (Labvisio the Autostainer 480)
4. Washed with PBS (Phosphate Buffered Saline) and to block endogenous peroxidase activity they were applied with 3% hydrogen peroxide for 10 minutes.
5. Washed with PBS twice in the preparation for protein blocking (BlockingReagent-Ultra V block, ofLabvisio) and they were performed with proteins blocking solution for 5 minutes.
6. Then washed twice with PBS, the primary antibody were dropped and incubated for 60 minutes.
7. After washing twice with PBS preparations were incubated with containing biotin secondary antibody for 45 minutes (Biotinylated Goat Anti-polyvalent, Labvisio's) .
8. Washing twice with PBS Strep-AB complex (Streptavidin Peroxidase,Labvisio's) dropped and allowed to stand for 45 minutes.
9. Washing twice with PBS, diaminobenzidine (DAB, Labvisio's) was used as a chromogen and the preparations were allowed to stand for 8 minutes after instillation.
10. Washing twice with PBS again Mayer's Hematoxylin (Merck Mayer'she malummicroscopysolution) was added drop wise and counter-staining was performed for 5 minutes waiting .
11. Washing twice with PBS automatically removed from the system and washed with tap water for 5 minutes and then dipped in alcohol for one time.
12. Were then dried and taken to xylene.
13. Then they were closed with entelan (Merck). As a positive control ; For CD3 and CD8 lymphoid hyperplasia had been diagnosed with the appropriate sections of tonsil, for FAS small bowel resection material selected from the normal intestinal tissue and appropriate sections were used.

Determining FASL: FASL antibody was used as the primary antibody (Neomarkers, Ltd.) .The appropriate sections from malignant melanoma samples were diagnosed as positive control.

1. 1.4mm thick sections were taken to lama adhesive. Preparation were left in the oven at 37 ° C overnight,
2. The next day was left in the oven for 20 minutes at 60 C, each time they were kept in xylene in twice for 10 minutes.
3. They were deparafinized after further keeping it in twice alcohols by 2 minutes. The sections were washed with distilled water.
4. Preparations for antigen release phase they were included in citrate solution (pH=6) at 98 ° C taking 23 minutes PT modulation (Labvisio, UK).
5. Preparations extracted from the module was allowed to cool. After washing with distilled water to block the endogenous peroxidase activity they were applied with 3% hydrogen peroxide for 5 minutes.
6. The preparations then washed with distilled water they were sequenced to Sequenz Manuel painting station (Shandon ScientificLimited, Astmo, UK). Phosphate buffer solution as the wash solution (Phosphate Buffered Saline-PBS) was used.
7. After washed twice the protein was blocked with protein blocking solution (Blocking Reagent-Ultra V block, Labvisio's) for 8 minutes.
8. After washing with PBS 2 times dropwise primary antibody were incubated in a humid environment for one night at room temperature.
9. The next day preparations which were washed 2 times with PBS were incubated for 45 minutes containing biotin secondary antibody (Biotinylated Goat Anti-polyvalent, Labvisio's).
10. After washed 2 times with PBS. Strep-AB complex (streptavidin-peroxidase, Labvisio's) dropped and waited for 45 minutes.
11. After washed 2 times with PBS. Diaminobenzidine (DAB, Labvisio's) was used as a chromogen and 10 minutes was expected after drop.
12. After washed 2 times with PBS. Mayer's hematoxylin (Merck Mayer's hemalum solution for microscopy) was added dropwise for 5 minutes and waited for counter-staining.
13. After washing 2 times with PBS they were washed with tap water for 5 minutes.
14. The preparations washed and they dipped in alcohol for one time.
15. Preparations after drying were obtained to xylene(Merck) and then closed with entelan .

CD3 and CD8 assessing immunoreactivity: For cytoplasmic staining was considered positive for both antibodies. 100 enterocytes in surface and in crypts were taken and the number of İEL were evaluated .Positively staining cells of 100 mononuclear cells were counted in the surface and in the deep the lamina propria.

Evaluation of FAS and FASLimmunoreactivity: Cytoplasmic staining was considered as positive in both of the immunedeterminants and modified immunohistochemical (H-SCORE) score was used. This scoring method have been described previously as HS=(Px L/ 100) (112) and İ as intensity of staining and Pİ as the number of stained cells. Using H-SCORE In all preparations in surface and crypt enterocytes, staining were evaluated separately in surface and İEL of crypts and in lamina propria mononuclear cells.

Statistical Review: SPSS 16.0 Windows statistical program was used. Groups contain less than 30 items and non-parametric tests were used because of the dispersion characteristics of data. To investigate the differences between groups were analyzed by Kruskal-Wallis.To determine statistically different groups the Mann-Whitney U test were evaluated.Correlation Spearman's correlation test was used. All tests were considered statistically significant at $p \leq 0.05$.

3. Results

3.1. Socio-Demographic Data

Adult study group included 29 CD (29.6%) cases , 17 NSD (17.3%) cases, 24 the group who has İEL in duedonum (24.5%). 28 units in normal duodenal mucosa biopsies used as K (control group) (28.6%) .İn CD group there was 21 women and 8 men and the mean age was 39.62±13.10. İn NSD group there was 10 women and 7 men and the mean age was 52.24 ±11.60. İn the group who has İEL in duedonum there was 15 women and 9 men and the mean age was 39.62±13.10. İn K group there was 20 women and 8 men and the mean age was 50.86±11.08.

Children study group included 7 CD 26.9 (%) cases, 6 cases as NSD (23.1%), 6 cases as the group who has İEL in duedonum (23.1%). 7 duodenal mucosal biopsy in the normal range used as theK group(26.9%) . İn CD group there was 4 women and 3 men and the mean age was 10.43±3.59. İn NSD group there was 5 women and 1men and the mean age was 13.50±2.42. İn the group who has İEL in duedonum there was 4 women and 2 men and the mean age was 14.83±1.47. İn K group there was 3 women and 4 men and the mean age was 11.86 ±4.67.

3.2. Immunohistochemical Results

IEL expressimg CD3 was detected by a greater number on the surface and in the crypts in HE sections. This number is significantly higher in CD, but gradually decreased in the group who has İEL in duedonum, NSD and in the K group.

3.3. Adult Group

CD3 and CD8 expression: When compared to NSD group , IEL with CD3 detected in surface and in the crypts was higher in the group who has İEL in duedonum but this difference was not statistically significant. There was significant difference between all other adults groups ($p < 0.001$). Number of CD3 positive cells in adult cases and mean values and standard deviations are given in Table3.

CD8 with IEL expressing in the surface and in the crypts,

though greater in number in the group who has İEL in duedonum when compared to the NSD group , although this difference did not reach statistical significance. Among all other groups, the difference was significant ($p \leq 0.001$).

In superficial lamina propria CD3-expressing mononucleer cells reach to highest levels in the celiac group and these values were higher than other three groups ($p < 0.001$). No statistically significant difference between the groups was observed. In superficial lamina propria CD8 expression of mononucleer cells was gradually decreased towards from the CD group to K group, NSD and the group who has İEL in duedonum. Statistically significant difference was observed between CD and K group ($p < 0.001$), the group who has İEL in duedonum and the K group ($p = 0.003$), NSD and the K group ($p = 0.028$).CD8 expression values of CD groups was significantly higher than both in the group who has İEL in duedonum and NSD group($p < 0.001$).

Deep in the lamina propria mononucleer cells maximum of CD3 expression was detected in CD and in the group who has İEL in duedonum.The values of these two groups were very close together. İELAG compared to theK group showed higher expression ($p = 0.050$). No differences between groups were observed. Again deep in the lamina propria mononucleer cells maximum of CD8 expression was detected in the group who has İEL in duedonum and in NSD group. Statistically no significant differences were observed between these two groups. CD8 expression in CD was higher than the K group ($p = 0.031$). The number of CD8 positive cells in adult cases and mean values and standard deviations are given in Table 4.

Table 1. *Number of CD3 positive cells in adult cases and mean values and the standard deviations*

	Surface İEL	Crypt İEL	Surface laminapropria	Deep lamina propria
K	11.21±4.85	2.39±1.34	22±6.36	45.89±11.45
CD	69±18.23	20.97±13.22	31.52±9.83	51.66±17.72
İEL*	25±5.26	8.17±4.17	19.96±5.87	51±9.42
NSD	22.82±4.17	7.06±3.50	19.65±5.02	47.06±8.66

İEL*:The group with İELin duedonum

Table 2. *Number of CD8 positive cells in adult cases and mean values and the standard deviations*

	Surface İEL	Crypt İEL	Surface lamina propria	Deep lamina propria
K	8.36±3.56	1.79±0.78	12.54±5.14	15.36±4.13
CD	58.21±15.34	12.41±8.15	24.59±8.29	19.86±7.45
İEL*	19.96±5.31	5.71±3.34	8.50±3.50	23.12±5.42
NSD	17.35±4.55	5.12±2.17	9.35±2.69	24.65±6.08

FASL expression: Evaluation of FASL expression in the surface and crypts of İEL, the highest H-SCORE values were detected in the group who has İEL in duedonum and NSD group. Statistically no significant differences were observed between these two groups.

H-SCORE valuesof the K group was significantly lower than both groups ($p < 0.001$). The crypt İEL expression in CD that was less significant than the group who has İEL in duedonum ($p = 0.007$) and NSD group ($p = 0.014$) but no significant difference was seen on the surface. Compared to the K group in CD H-SCORE values were higher ($p < 0.001$).

In the lamina propria: The highest FASL H-SCORES values reached in the group who has İEL in duedonum in mononucleer cells in the lamina propria. FASL H-SCORES was lower in NSD group than the group who has İEL in duedonum but the difference was not statistically significant. Compared to the K group CD H-SCORE values were higher, but not statistically significant.

In surface and crypt enterocytes: The highest FASL H-SCORES was in the group who has İEL in duedonum in surface and crypt enterocytes. However this values seem like higher compared to the NSD groups ,it was not statistically significant. But, in both groups compared to the K group H-SCORES were significantly higher ($p < 0.001$).

İt was noticed that the both in surface and crypt enterocytes in CD H-SCORES were less than the group who has İEL in duedonum ($p < 0.001$). Similarly significance were detected in surface of the enterocytes ($p = 0.001$) and crypt enterocytes ($p < 0.001$) between CD and NSD group.

Compared with the K group higher H-SCORES were detected in the CD in surface enterocytes but difference was not significant, higher values close to significance($p = 0.057$), are obtained in crypt enterocytes (Table 5).

Table 3. *Mean values and standard deviations in adults with FASL H-SCORES*

	Surface İEL	Crypt İEL	Surface enterocyte	Crypt enterocyte	Lamina propria
K	104.14±69.44	98.75±71.21	137.07±76.09	110.29±77.14	101.68±67.12
CD	199.14±55.11	182.41±64.83	169.17±57.07	142.14±67.02	121.14±43.66
İEL*	216±48.62	225.21±46.49	235.33±33.99	234.67±38.57	203.08±46.43
NSD	207.24±59.06	225.76±42.75	221.65±38.81	219.12±39.30	188.71±38.20

Table 4. *Mean values and standard deviations in adults with FAS H-SCORES*

	Surface İEL	Crypt İEL	Surface enterocyte	Crypt enterocyte	Lamina propria
K	65.07±45.05	100.86±39.67	142.86±57.36	194.57±54.64	89.89±52.88
CD	225.46±75.24	198.93±61.23	249.75±54.41	222.25±46.38	169.54±49.27
İEL*	65.25±41.64	81.25±47.33	153.67±51.34	195.88±58.57	83.29±38.12
NSD	99.41±50.01	114.47±56.07	173.18±51.37	212.65±38.89	95.12±31.09

FAS Expression:

In surface and crypt enterocytes: The highest FAS H-SCORE in surface and crypt enterocytes was belonged to CD. This group was followed in descending order as NSD, the group who has İEL in duedonum and K group. Significant difference ($p < 0.001$) viewed between CD and K group in surface enterocytes but the difference in crypt enterocytes were close to significance ($p = 0.065$). CD group compared to the the group who has İEL in duedonum and NSD, FAS H-SCORE was higher in the surface enterocytes ($p < 0.001$) and no significant difference was observed in the crypt enterocytes. Surface and crypt enterocytes, among other groups showed no significant difference in the expression of FAS.

In surface and crypts İEL: FAS values in surface İEL in CD was found to be significantly higher in all adult groups ($p < 0.001$). İn NSD group FAS expression on surface İEL was statically significantly higher when compared to K group ($p = 0.011$) and the group who has İEL in duedonum ($p = 0.036$).The highest FAS H-SCORE in crypt İEL was belonging to CD and from all groups this was statistically significantly higher ($p < 0.001$). While there was no significant difference between the K group and the group who has İEL in duedonum and NSD group ,the difference between NSD group and the group who has İEL in duedonum was close to significance ($p = 0.052$).

In the lamina propria: İt was detected that FAS values in the lamina propria mononucleer cells are higher in CD than other groups ($p < 0.001$). There was no difference between the other groups.

3.4. According to Spearman's Correlation Test

Positive correlation was found between CD3 and CD8 in CD with the number of İEL in surface ($r = 0.925$, $p < 0.001$) and crypts ($r = 0.955$, $p < 0.001$). Also there was positive correlation with CD3 and CD8 ($r = 0.589$, $p < 0.001$)in the deep lamina propria mononucleer cells, no correlation was found in the superficial lamina propria.

Positive correlation was found between CD3 and CD8 in the group who has İEL in duedonum with the number of İEL in surface ($r = 0.867$ $p < 0.001$) and crypts($r = 0.918$ $p < 0.001$). Also there was positive correlation between number of mononucleer cells in superficial lamina propria with CD3 and CD8 ($r = 0.668$, $p < 0.001$); deep in the lamina propria there was no such relationship.

Positive correlation was found between CD3 and CD8 in NSD group with the number of İEL in surface ($r = 0.665$ $p = 0.004$) and crypts ($r = 0.538$ $p = 0.026$) . There was positive correlation detected between CD3 and CD8 in surface ($r = 0.521$, $p = 0.032$) and deep ($r = 0.484$, $p = 0.049$) mononucleer cells in the lamina propria.

Positive correlation was found between CD3 and CD8 in K group with the number of İEL in surface ($r = 0.922$ $p < 0.001$) and crypts ($r = 0.804$ $p < 0.001$) . Also positive correlation between number of mononucleer cells in superficial lamina propria and CD3 and CD8($r = 0.531$, $p = 0.004$); deep in the lamina propria there was no such relationship.

3.5. Results belonging to Childrengroup

CD3 and CD8 expression: The number of CD3 and CD8 positive cells on surface ($p = 0.002$) and crypts ($p = 0.001$) in CD were found to be higher than K group. Again in CD compared to the group who has İEL in duedonum higher number of CD3 ($p = 0.004$) and CD8 ($p = 0.008$) was detected on surface but observed no significant differences in the crypts. CD3 and CD8 with number of IEL on surface was significantly higher in CD than NSD group ($p = 0.003$) but no significant difference was detected in the crypts.

When the group who has İEL in duedonum compared with the K group; the number of CD3 ($p = 0.002$) and CD8 positive cells ($p = 0.003$) in both surface and crypt epithelium were found to be higher. NSD group compared to the K group, CD3 ($p = 0.004$) and CD8 ($p = 0.005$) with number of IEL on surface were determined higher.There was significant difference in the crypts when lymphocytes counted by immun markers($p = 0.002$). Between the group who has İEL in duedonum and NSD group significant difference ($p = 0.004$) was observed with the number of IEL on surface and CD3 and CD8-positive cells ($p = 0.004$)but there was no difference in the crypts.

In the lamina propria: CD3 expression of superficial lamina propria mononucleer cells were to come to the highest values in children with CD. Although expression was higher in NSD when compared to K group, it did not reach statistically significant value. Expression was detected at least in the group who has İEL in duedonum. There was significance between the group who has İEL in duedonum and CD ($p = 0.010$) and close to significance between in NSD group and the group who has İEL in duedonum ($p = 0.053$). There was no statically significant difference between the other groups.

The more positivity of CD8 in superficial lamina propria was in CD.CD8 expression was significantly increased in children with CD compared to K group and the group who has İEL in duedonum ($p = 0.003$) and NSD group ($p = 0.002$).

Deep in the lamina propria the most CD3 positive was in NSD group and the least positiveness was found in CD. NSD and the CD group ($p = 0.010$) were significantly different. The expression was higher in the group who has İEL in

duedonum when compared to the K group (p = 0.038) and CD (p = 0.007). When analyzing CD8 expression in mononuclear cells in the deep lamina propria significance was found between K group and CD group (p = 0.046) ;also between the group who has İEL in duedonum (p = 0.025) and NSD group (p = 0.021). There were no differences between groups.

Table 5. *Mean values and standard deviations in children with FASL H-SCORES*

	Surface İEL	Crypt İEL	Surface enterocyte	Crypt enterocyte	Lamina propria
K	117.29±71.43	116.14±80.04	137.71±59.54	131±65.17	112.57±55.87
CD	222.29±45.28	202.57±38.16	177.71±38.87	152.86±75.82	167.43±66.18
İEL*	245.17±43.73	247.50±32.78	231.83±42.29	228.17±27.27	208.83±55.54
NSD	250.67±27.52	261.83±18.26	229.33±34.47	244.33±27.80	247.33±16.53

FASL Expression:

On surface and in the crypts: Highest values were in NSD group, the lowest values were found in the K group. Values were found statistically significantly reduced in surface FASL in the K group when compared to CD group (p = 0.015), the group who has İEL in duedonum (p = 0.007) and NSD group (p = 0.010).FASL of crypts in the K group was with decreased values when compared to CD group (p = 0.035), the group who has İEL in duedonum (p = 0.010) and NSD group (p = 0.003) . Again the group who has İEL in duedonum with CD (p = 0.027) and NSD group (p = 0.003) were significantly different from other.

FASL expression, on the surface of enterocytes in the group who has İEL in duedonum (p = 0.032) and NSD group (p = 0.022) compared to K group was significantly higher. In addition, (p = 0.026) decreasing was viewed significantly in CD compared to NSD group. Similarly increasing was found significantly on the surface of enterocytes and in the crypts of the group who has İEL in duedonum (p = 0.015) and NSD than the K group (p = 0.010). FASL expression of CD group was significantly less than NSD group (p = 0.022).

In the lamina propria: FASL expression in the lamina propria was maximun in NSD group and minimum in K group. A statically significant increase in FASL expression was noticed in the group who has İEL in duedonum (p = 0.022) and NSD group (p = 0.003) when compared to K group. Again, expression was even more in NSD than CD group (p = 0.010).

FAS expression:

On surface and crypt enterocytes: The highest values on the surface were with CD group, the lowest values were detected in K group. Statistically significant differences were between CD and K group (p = 0.002), the group who has İEL in duedonum (p = 0.003) and NSD group. (p = 0.003)

The highest values in the crypts were again with CD group, the lowest value was found in K group. There were similar scores between the group who has İEL in duedonum and NSD group. Statistically no significant differences between groups were observed.

Surface and in the crypts İEL: FAS expression in İEL on surface and crypts are maximum in CD and minimum in K group. Although more expression observed in the group who has İEL in duedonum than in NSD group the difference was not statistically significant. Reaching statistically significant differences was in form like; - between CD and K group on surface (p = 0.002) and in crypts (p = 0.003),- between the group who has İEL in duedonum and K group on surface (p = 0.026) and in crypts (p = 0.027),-between CD and the group who has İEL in duedonum on surface (p = 0.003) and in crypts (p = 0.010),-between CD and NSD group on surface (p = 0.003) and in crypts (p = 0.007).

In the lamina propria: Expression in mononuclear cells was found highest in CD group, the lowest expression in K group. Altough scores are higher in the group who has İEL in duedonum than NSD group, but this difference was not significant. Between CD group with K group (p = 0.003) and İELAG (p = 0.003); and between the group who has İEL in duedonum with CD (p = 0.046) and NSD group (p = 0.012) there were statistically significant differences observed.

Table 6. *Mean values and standard deviations in children with FAS H-SCORES*

	Surface İEL	Crypt İEL	Surface enterocyte	Crypt enterocyte	Lamina propria
K	65.86±42.40	85.29±47.13	138.43±19.22	192.86±56.52	71.71±27.38
CD	259.29±37.99	225.29±46.33	281.86±5.24	239.29±41.24	192.29±44.74
İEL*	130.17±30.56	144±31.62	187.83±51.98	203.50±56.69	141.67±19.78
NSD	85.83±52.19	102.83±55.31	174.83±51.35	218.50±53.68	110.83±44.97

3.6. Spearman's Correlation Test

There was a positive correlation between CD3 and CD8 with the number of IEL in CD on surface (r = 0.889, p = 0.007) and crypts (r = 0.836, p = 0.019). Negative relationship was found in superficial lamina propria mononuclear cells between CD3 and CD8(r = -0,844, p = 0.017), but no correlation was found deep in the lamina propria. Positive correlation was detected between CD8 expression and FASL expression in İEL on the surface (r = 0.778, p = 0.039) found no correlation between CD3 and FASL. FASL and FAS expression in crypt enterocytes (r = -0,786, p = 0.036) were negatively correlated.

There was a positive correlation between CD3 and CD8

with the number of IEL in the group who has İEL in duedonum on surface (r = 0.841 p = 0.036) and crypts (r = 0.955 p = 0.003). Also positive correlation between CD3 and CD8 and number of mononucleer cells in the deep lamina propria (r = 0.841, p = 0.036), no correlation was found in the superficial lamina propria.

İn the crypts of NSD group (r = 0.955 p = 0.003) a positive relationship was found between with the number IEL and CD3 and CD8, the relationship was not observed on surface. Negative correlation was found in crypts of İEL with CD3 expression and FAS expression (r = -0,986, p <0.001) and with CD8 expression and FAS expression (r = -0,941, p = 0.005). Positive correlation was found between FAS expression and FASL expression on the surface of enterocytes (r = 0.829, p = 0.042).

On the surface in the K group (r = 0.924 p = 0.003) with the number of CD3 and CD8 of IEL positive correlation was found. Positive correlation was found between CD3 and CD8 (r = 0.826, p = 0.022) and the number of superficial lamina propria mononuclear cells, no correlation was found deep in the lamina propria.

4. Discussion

Environmental factors interact with genetic predisposition and immunologic factors resulting etiopathogenesis complex of CD(9). The mechanisms leading to the development of mucosal atrophy is not yet fully understood, but with matrix metalloproteinases destructuring and excessive apoptotic loss of enterocytes are likely the most effective mechanisms (21,22). FAS-FASL system which induces apoptosis is one of the most important pathways (23).İn the literature for adults and children with CD supporting studies are available that FAS-FASL system is involved in the apoptotic process (20,24). However, there are no studies that evaluated together about FAS-FASL expression in CD, NSD and the group who has İEL in duedonum. İn this study significance of FAS-FASL expression is investigated in patients the group who has İEL in duedonum and NSD to distinct from CD.

There are publications suggesting that CD3 immunohistochemistry would help in the evaluation of İEL (25,26). In all groups of our study the majority of İEL was constituted from lymphocytes expressing CD3. Oberhuber et al. have shown likewise that; the majority of İEL in CD ,in giardiasis and normal duodenal mucosa shows CD3 positiveness (27).The number of İEL with CD3-positive enterocytes on the surface was higher in all groups when compared in crypts. Similar results shown in the literature in the jejunum mucosa (28) and in duodenal mucosa (29) in CD and in normal individuals .We observed that the number of CD3-positive cells onsurface and in crypts are reduced towards the CD from the group who has İEL in duedonum, and group of NSD and K group. CD8 positive cells gave similar results with the number of CD3. Our findings showed that predominant subtype in İEL were CD8 positive cells both in CD and normals and such study was done by Selby (30) and Verkasalo et al (30) in jejunum mucosa. Again, whether H. pylori gastritis patients the majority of İEL in duodenal mucosa were detected as CD3 and CD8 positive(31). In our study, all groups in adults there was a positive relationship between CD3 and CD8 expression on surface and crypt enterocytes.In cases of NSD in children on the surface İEL and in the crypts İEL of K group we could not determine positive relation. This results in children thought as caused by the small number of patients. Line with our findings Hasan et al. found that the number of IEL increased in cases of NSD compared to the K group (32).

In our study we also assessed CD3 and CD8 expression of superficial and deep lamina propria mononucleer cells . Both adults and children in all groups the number of CD3-positive cells were determined higher in the deep lamina propria than superficial area.In this case, superficial lamina propria plasma cells are thought to be the cause of condensation. Unlike the expression of CD8 in all groups and this difference is also significantly higher in both children and adults in CD in superficial lamina propria than deep lamina propria. We suggest that in CD cytolytic activity in the pathogenesis of the disease and this plays an important role in mucosal damage.

In our study,CD8 expression in the superficial and deep mucosal mononucleer cells were similar both in K group of adults and children;but there was significantly higher expression in the group who has İEL in duedonum and NSD group in the deep lamina propria.The number of CD3 positive cells were higher in deep lamina propria than surface in the group who has İEL in duedonum and NSD group and this suggests that T cells are concentrated in the deep lamina propria in inflammatory processes when compared to normal mucosa.

FAS, which induces apoptosis is a member of the TNFR family (33). İt is expressed wide variety of tissues and in the organ (34). Crypt-villus axis in the small intestine and colon epithelium along located in the basolateral surface of the cells. In a study in vitro of epithelial colonic crypts when incubated with agonists FAS antibodies it goes to apopitosis. However, this functional FAS expression in the normal gastrointestinal tract and it is biological significance is unclear (35).

In our study, we've detected FAS expression in enterocytes in all groups of adults and children. Ehrman et al. showed FAS expression in all groups of Celiac children , potential celiac and control group (24). Similarly Ciccocioppo et al. showed FAS expression in all of the adults with CD, group who CD has been treated and control groups (20).The same researchers, by the method of cell isolation system; they showed FAS expression in enterocytes in active CD is significantly higher than patients treated and control group (7). Maiuri et al. found high FAS expression in enterocytes and this was associated with villous atrophy (11). Ciccocioppo et al. showed that FAS expression is limited to a few enterocytes in normal duodenal biopsies, but FAS expression in CD in crypts is common both in the mucosa and on surface (25). In our study, both children and adults in CD group compared to the K group on surface and crypt

enterocytes ,FAS expression was found significantly higher in CD group.This also suggests that FAS-mediated cell death caused by loss of enterocytes may play a role in the developing of villous atrophy in CD.

Ehrman et al. found that FAS expression of enterocytes in the crypts of all groups of children is higher according to the surface (24). Similarly Ciccocioppo et al. showed that FAS expression in all groups in adults of crypt enterocytes greater than those of the surface (10).However, this uses the materials and methods of research people are different from ours. In our study, in CD in the crypt enterocytes FAS expression was more than surface enterocytes but in all other groups expression was mostly found on surface than crypts. İf these differences we detected in CD is supported by other studies this can also help in the diagnosis of CD as an identifier. Ehrmann et al. fouNd that FAS positive enterocytes in jejunum biopsies of children significantly lower in active CD compared to potencially CD and control groups, the percentage of FAS positive lymphocytes in mucosa was lower in active CD compared to potencially CD(24). Ehrmann et al's study, the detection of these findings contradicts our datas as well as with other relevant literature. Di Sabatino et al. showed that FAS expression was significantly higher in peripheral blood lymphocytes of CD with untreated and treated patients than the control group (36). In our study, we detected that FAS expression scores in mucosal lymphocytes on surface and crypt İEL in CD were significantly higher than the K group.We've found high FAS expression in mucosa and intraepithelial lymphocytes in CD and this can be a regulatory mechanism seen in the CD which control activation of both mucosal and peripheral lymphocytes .

In our study FAS and FASL expression in İEL was seen in adults and children K group. The functional significance of this expression is not known. Hongo et al. showed that isolated İEL from human colon tissue has almost strong expression of FAS, FASL expression in large part, a small fraction of peripheral blood mononuclear lymphocytes expresses FAS and FASL very lower.In contrast these expressions are active in the development of apoptosis in İEL in peripheral blood mononuclear cells ; it is thought FAS-FASL expression to have functional effects in the regulation of lymphocyte homeostasis and immune responses in the intestine which exposed to continuously as complex mixtures of microorganisms and nutrient content of intense antigenic stimulation(37).

Our study shows an increase in IEL in children and adults in the duodenal mucosa and NSD group compared to the K group and FAS expression on surface and crypt enterocytes was found to be higher. However, this difference was not statistically significant. Expression was lower in both groups than in CD.This statistical difference in surface enterocytes in both adults ($p < 0.001$) and children ($p = 0.003$) were significant. In the literature supporting that inflammatory changes with FAS-FASL pathway in the gastrointestinal tract starring in the pathogenesis of CD and as well as in GVHD (38,39), IBD (inflammatory bowel disease) (14,15), Helicobacter pylori infection. GVHD is of importance because of the diseases of the gastrointestinal tract the main target of this disease is damage to the intestinal epithelium (9). T cell activation is the key step in the pathogenesis of CD, GVHD as idiopathic İBD and other inflammatory diseases of the intestine. In the formation of the inflammatory response, activated T cells are involved in both regulatory and effector mechanisms. Effector mechanisms which defense against microorganisms are important cause of the formation of the significant tissue damage. Flattening mucosa and intestinal enterocyte apoptosisis recognized as the major mark of T cell-mediated tissue damage in inflammatory diseases . In this sense, Merger and colleagues worked in vivo in mice by forming mucosal damage with CD3 antibody in small intestine and they showed that T cell activation can cause enterocyte apoptosis and villous flattening. Then the formation of tissue damage induced by T cell activation, in which especially perforin,TNF-alfa and FAS joined and this mediated in many pathway as a combined effect (13). Similarly Braun et al. (41) they worked with mice and showed that perforin and FAS pathways roles in tissue injury. Waseda et al. demonstrated that in donor T cells ; FAS precursors which occur with FASL accumulation and activation involved in the pathogenesis of the disease (40).

Ueyama et al. showed that there was FAS expression in ulcerative colitis and Crohn's disease in frozen sections and expression was in diffuse areas of colon tissue both in normal and ulcerative colitis. Inflammatory cells also showed weak immunoreactivity. They also showed FAS expression in Crohn's disease and inactive ulcerative colitis but did not specify the data (14). Iwamoto et al. identified that FAS and FASL expression increase in epithelium and lamina propria in ulcerative colitis compared to normal colonic mucosa, and most of these cells are TUNEL positive showing that FAS-FASL interaction is the mediator of apoptosisin ulcerative colitis (15).

İn gastrointestinal tract FAS / FASL pathway is thought to have a role in pathology of Helicobacter pylori infection. Gargal et al. showed in their study that with patients who has functional dyspepsia and in duodenal mucosa of these, the number of IEL expressing FAS was significantly higher in H. pylori positive patients than the H. pylori negative patients and control group (42). In the gastric antrum and corpus mucosa in H. pylori-related gastritis FAS / FASL expression is increased and there are studies showing the relationship in apoptosis in adults and children (29-31).

İn GVHD, IBD and Helicobacter pylori infection, such inflammatory processes seen in the gastrointestinal tract; in our study we saw increasing of FAS expression in the group who has İEL in duedonum and in NSD than normal mucosa. FAS-FASL expression is therefore not an effective mechanism in the pathogenesis of CD but very high FAS expression may be associated with CD.FASL is type II integral membrane protein of the TNF family (43). FASL binding to the extracellular domain of the FAS provides trimerization of receptor and this starts apoptotic process. In

previous years thought that FASL expression is limited with activated T and natural killer (NK, natural killer) cells ; in recent years in studies expression is reported in different human cells and tissues in lung, liver and kidney (43). However, there are doubts about the specificity and sensitivity of the antibody that is used on FASL (44). Therefore some investigators used multiple different FASL antibody to see to support the reliability of the results (45.46).

Melgar et al. demonstrated the role of FAS-FASL pathway in FAS expression in villi and crypts İEL in the normal jejunum mucosa and lamina propria and appearance of cytotoxic activity of T lymphocytes. Killing through of T cells in the normally jejunum mucosa by other T cells with FAS-FASL pathway this may role in local tolerance against antigens in the diet, in elimination of T cells showing ekstratimik maturation and removal of infected cells with virus (47). This study we detected FASL expression in normal tissues can have functional significance and it should be investigated.

Previously the principal authors reported that FASL expression in gastric mucosa is associated with Helicobacter pylori infection (48,49) but Kotlowsk-Kmiec et al. in their study in recent years they showed that inflammatory changes in the mucosa in the stomach which is not accompanied by Helicobacter pylori infection may lead to an increase in FASL expression (8). These findings in our study,supports that FASL expression is increased in in inflammatory changes with unknown etiology .

CD studies showing increased FASL expression is available (11,12). Ciccocioppo et al. showed that almost all of İEL is FASL positive in active CD, this value is significantly higher when compared to treated CD and control groups (11). Maiuri et al.found that FASL expression in İEL in untreated CD patients was similar to cases with villous atrophy and with normal villous morphology; but in patients treated as CD and the control group were almost negative (11). Ciccocioppo et al.found that FASL expression in İEL in the duodenal mucosa and lamina propria mononucleer cells were significantly higher in CD than control group (12).

In our study, in adults and children in the CD group ,the FASL expression ons urface and in crypts in İEL was found to be higher than K group. This difference is statistically significant in both children and adults on surface and in crypts. Ehrmann et al. showed that the number of FASL expressing İEL is very low in active CD , potential CD and the control group and there was no difference between groups. The proportion of mucosal lymphocytes expressing FASL were found to be significantly higher in active CD (80%) and potentially CD (60%) than the control group (28.6%) (24).

İn our study in children and adults with CD, FASL expression was found to be higher in mucosal lymphocytes compared to control group, but the difference was not statistically significant. Ciccocioppo et al. found that FASL positive lamina propria mononuclear cells in untreated CD is significantly higher than the control group, this decreased after treatment but not return to normal (10).

Ehrmann et al. found that the rate of FAS positive enterocytes in all groups was over 80% in half of the upper villi. In the crypts of active CD (84%) and potential CD(86.7%)FASL positive enterocytes were higher than the control group (76.2%) (24).

In our study, FASL scores on surface of enterocytes in children and adults were found to be higher than the crypt enterocytes. FASL scores were higher In surface and crypt enterocytes in children and adults in CD than the Kgroup. FASL expression on surface and crypt enterocytes were similar in the group who has İEL in duedonum and NSD group of children and adults,on surface and crypts İEL, in lamina propria mononuclear cells and these values were significantly higher than the CD and K group.There was no statistically significant difference between the two groups. These parameters have detected a statistically significant difference both in child and adult group with the group who has İEL in duedonum and NSD group than the Kgroup .

Up to our study we do not know the importance of FASL expression which we've found higher in the group who has İEL in duedonum and NSD patients than CD. FAS-FASL apoptotic pathway plays a role in both enterocytes apoptosis and in T lymphocytes apoptotic death. Finding excessive FASL expression in the unknown etiology of the group who has İEL in duedonum and NSD group it might be the indicator that FASL is more sensitive to inflammatory changes, and perhaps clearance of proliferating lymphocytes in the inflammation area is stronger in inflammatory process than CD; than the complications that can develop in CD (refractory celiac disease) inhibited by clonal proliferation of lymphocytes. However,demonstration of FAS and FASL-expressing cells in diseased tissue is not enough to show that FAS-mediated cell death involved in the incident. All FAS-expressing cells are not sensitive to FAS-mediated cell death. There are many protective mechanisms to defend from apopitosis. IAP (inhibitor of Apoptosis Protein), FLIP (FLICE-inhibitory protein) and decoy receptors, supresses cell death mediated by FAS with various mechanisms. So when evaluating FAS-mediated apoptosis it is reported to be more appropriate to show whether FAS positive cells are sensitive to death with anti-FAS monoclonal antibody and use of cross-linked SFASL. Similarly FASL presence in the cell membrane does not mean that FASL expression is necessarily capable ofmediating killing of FAS-sensitive target cell. In certain tissues, depending on the type of tissue,it can soluble by membrane FASL metalloproteases and can degradable to less potencially cytotoxic form (9). Therefore, in these cases we've found that inhibitory mechanisms are activated in regulation of apoptosis to prevent mucosal damage against high FASL expression in enterocytes.

We think that in all groups to determine the functional significance of FAS-FASL expression , a method of assessing apoptosis needs to be included in the study and this will be useful.

Different results were obtained from studies evaluating

correlation of FAS and FASL expression of lymphocyte subtypes. We did not find work in the literature about dealing with this issue in duodenal biopsies. However in Kotlowsk A-Kmiec et al. study of Helicobacter pylori gastritis in the gastric mucosa ,only positive correlation between CD4 and FAS expression was found; but no correlation between CD3, CD8 and CD20 with FAS and FASL expression (18).Ibuki et al. determined positive correlation between CD8 positive T cells and FASL positive cells in portal inflammation, interphase hepatitis and focal necrosis areas in viral hepatitis (50). Di Sabatino et al.demonstrated that there was FASL expression in both CD4 and CD8 positive cells in the peripheral blood of patients with celiac, FAS expression in the CD8 positive cells were to be significantly higher than control (36).In our study, children in the CD group we found a positive relationship between CD8e xpression and FASL expression on surface İEL , that supports the role of CD8-positive lymphocytesin FAS-FASL pathway.

In studies with CD, cells interaction was investigated in apoptosis and than correlation methods assessed between FAS-FASL expression (10-12).We believe that the correlation evaluation is strong and a portion of the FAS and Fas-expressing cells will lost in apoptotic process.

We detected a statistically significant difference only in children group with NSD and the group who has İEL in duedonum, between the number of IEL and CD3 and CD8-positive cells on surface and in adults with FAS expression on surface İEL.

As a result in inflammatory process in the small intestine FAS-FASL expression is increased compared to normal and FAS-FasL expression can not help in the discrimination of these pathologies.

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