MODULATION OF β_2 AND β_3 INTEGRINS IN EXPERIMENTAL COLITIS INDUCED BY IODOACETAMIDE AND ENTEROPATHOGENIC *E. COLI*

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Integrins can modulate the infiltration of inflammatory cells and the secretion of various inflammatory mediators, essential players in the pathogenesis of colitis. This study explores the role of β_2 and β_3 integrin signaling and their possible role in experimental colitis. A total of 160 adult male Sprague-Dawly rats were divided into 4 equal groups: methylcellulose, bacteria, iodoacetamide and iodoacetamide plus bacteria. Clinical symptoms and signs of colitis were checked daily and colonic tissues were biopsied on days 3, 14, 28, and 56 post induction. Histological studies along with histochemical analysis and polymerase chain reaction of β_2, β_3 and $\alpha_v\beta_3$ were performed according to standard procedures. The symptoms and signs were consistent with previously reported data on active colitis. The highest expression of β_3 integrin was in the combined treatment mostly on platelets, endothelial and inflammatory cells. In the same group, the expression of $\alpha_v\beta_3$ integrin complex reached the highest score after 56 days in all colonic layers. β_2 integrin expression showed a 3-4-fold increase in the combined treatment group at all time points and kept increasing till day 56. It was mostly expressed in the mucosa and submucosa. In addition, the expression of both $\alpha v\beta_3$ and $\alpha_{ii}\beta_3$ integrins was also elevated 2- to 10-fold, respectively, in the same colitis groups throughout the duration of the experiment. In conclusion, the combined treatment of IA and Enteropathogenic *E. coli* led to a significant upregulation of all the tested integrins throughout the experimental duration. Such upregulation of integrins could have contributed to the increase and chronicity of inflammation.

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Remarkable progress has been made in the study of the pathogenesis of inflammatory bowel disease (IBD), either ulcerative colitis (UC) or Crohn's disease (CD), using animal models. More than 63 models have been developed in the past 30 years including the iodoacetamide (IA) and the enteropathogenic *E. coli* (EPEC)-induced model reported by our group (1, 2). Such models have increased our understanding of the underlying pathogenesis and helped to delineate the many factors, cofactors, and etiologic agents involved in the common, non-specific inflammatory processes that result in tissue injury and the periodic remitting and relapsing inflammation, both hallmarks of IBD (1-3).

Multiple etiologic factors are involved in the

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pathogenesis of IBD including the intestinal flora, chemicals, diet, the mucosal immune cells and other molecules like integrins (3, 4). Some of these factors could lead to a significant increase in the mucosal infiltration of inflammatory cells, particularly leukocytes, and to the secretion of a cascade of various mediators or substances produced by the diverse cellular constituents present at the sites of disease activity or in distant locations (4-7). One approach to the management of such disease entities is to specifically inhibit individual mediators or the migration of leukocytes into the inflamed intestinal tissue by blocking cellular adhesion molecules like integrins (8, 9). Integrins constitute a superfamily of at least 24 identified αβ pairs of heterodimeric proteins. They play a major role in cellular adhesion, migration and proliferation, starting as early as embryonic life and their expression is altered in IBD's (8-10).

On the other hand, the gastrointestinal tract is constantly challenged by foreign antigens and commensal bacteria, but nonetheless is able to maintain a state of immunological quiescence (5, 11). Recent advances have highlighted the importance of active suppression by regulatory lymphocytes and immunosuppressive cytokines, and the central role of some integrins in controlling mucosal immunity and maintaining normal regulation of immune responses in the intestine (5, 10-13).

As a result of their functions in migration, infiltration, and "homing" of leukocytes, many integrins have been implicated in the pathogenesis of IBD. Both UC and CD are accompanied by upregulation of certain integrins with an elevated production of inflammatory mediators to induce inflammation as well as tissue injury (1, 2, 6). The main integrins involved are β_3 ($\alpha_v\beta_3$ and $\alpha_{11b}\beta_3$), αv (αvβ5, $\alpha v\beta_8$), β_7 and β_2 (4, 7-9, 11-13). Different integrins participate in different ways. In a murine model of colitis, it was shown that the blockade of the murine angiogenic endothelial marker $\alpha_v\beta_3$ decreased effectively both neoangiogenesis and inflammation (13-15).

Integrins can also contribute to cancer progression by influencing processes such as metastasis, induced platelet aggregation, angiogenesis, and vascular wall apoptosis. It was reported that cancer is associated with the dysfunction of β_3 integrin and that an inhibition of $\alpha_v\beta_3$ expression inhibits tumor formation (16-19). It is important to note that $\alpha_v\beta_3$ can also be found in active and inactive forms, just like $\alpha_{11b}\beta_{3,}$ which is one of the cell adhesion receptors and has a more restricted distribution than $\alpha_v\beta_3$. $\alpha_{11b}\beta_3$is mostly expressed on platelets and plays a crucial role in homeostasis, vascular functions and adhesion. Pharmacologic inhibitors to $\alpha_{11b}\beta_3$ have been developed and are widely used to prevent coronary thrombosis (7, 18, 19). On the other hand, αv was shown to play a primary role in the healing of epithelial tissues (20). It was also demonstrated that the pleiotrophic role of α_v integrin was central to the normal regulation of immune responses in the intestine and that deletion of α_v leads to spontaneous colitis, wasting, autoimmunity, cancer in mice, and impaired removal of apoptotic cells (20). The major infiltrating cells in UC: macrophages; dendritic cells and neutrophils are the major expressors of the α_v integrin (12 -15).

Further studies suggested a dual role for α_v integrins. $\alpha_v\beta_3$ and $\alpha_v\beta_5$ mediated the uptake of apoptotic cells by macrophages and dendritic cells, thus, providing self-antigen and modifying the extent of inflammation. In addition, $\alpha_v\beta_8$ generated regulatory T cells through local activation of TGFβ and conditioning of dendritic cells (12). S247 antagonist targeted $\alpha_v\beta_3$, and inhibited tumor angiogenesis, increased endothelial cell apoptosis, and decreased pericyte coverage. It also inhibited leukocyte trafficking, the growth of primary tumors and their metastasis (7, 16).

It is also important to note that $\alpha_4\beta_7$ integrin is primarily involved in the recruitment of leukocytes to the gut. It is present on the cell surface of a small population of circulating T lymphocytes, primarily involved in the control of trafficking of lymphocytes to the intestine and their activation, very much like β_2 integrins (9). The β_2 integrin is also expressed in all layers of the normal intestine and colon with a role in maintaining normal intestinal homeostasis. It has also been reported to increase in UC and CD (13).

Recent studies in our laboratory using an IBD model induced by IA and EPEC, showed the dependence of the inflammatory activity on various individual soluble mediators and cell adhesion molecules capable of reducing the integrity of the

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epithelial barrier, and recruiting inflammatory cells from the vascular compartment to the site of disease activity (1, 2).

The role of integrin signaling in mucosal inflammation is presently not very well known. Using the above-mentioned model, we report the modulation of expression of various integrins; β_2 (exclusively expressed on white blood cells migrated to the gut), β_3 (expressed on new blood vessels coupled to α_v and on platelets coupled to α_{IIb}) and $\alpha_v\beta_3$ (exclusively expressed on newly formed blood vessels) and their possible role in the pathogenesis of chronic colitis.

MATERIALS AND METHODS

Animals

A total of 160 adult male Sprague-Dawley rats, weight range 200±25 g, were used in this experiment in accordance with the criteria set for care and use of animals by the Institutional Animal Care and Use Committee at the American University of Beirut. Animals were housed in rack-mounted cages, with a maximum of 10 rats per cage, and kept on a 12 hours light/dark cycle in a controlled temperature and humidity room. Standard laboratory pelleted formula and tap water were provided ad libitum.

Induction of experimental colitis

The methodology of induction was identical to previously reported work (1). The rats were randomized into 4 groups of 40 rats each and inoculated intrarectally on a weekly basis, unless otherwise specified, for 56 days: (i) the methylcellulose (MC) treated control group, a 100 µl of 1% MC, the vehicle (Sigma, M-0512, USA); (ii) the iodoacetamide-treated (IA) group whereby the rats were inoculated with 100 µl of 6% IA (Sigma, 1-6125, USA) based on the previously described experimental model by Satoh et al. in 1997 (3); (iii) the bacteria-treated (B) group was inoculated with 200 µl suspension containing $4x10^8$ colony factor unit of EPEC bacteria; (iv) the combined treatment group, whereby a combination of IA was followed by B after 48 hours. On day 28, however, each group was split into 2 subgroups. In one subgroup inoculation of the different treatments continued as preplanned, and in the second subgroup it was discontinued. Six rats from each group and later subgroup (after 30 days), at each time point, were anesthetized by intraperitoneal injection of sodium pentobarbital (75 mg/kg body weight) and sacrificed on days 3, 14, 28 and 56. The descending colon tissues were immediately washed in cold physiological saline, a part was fixed in 10% buffered formalin for routine microscopy, and colonic mucosa scrapings were snap frozen for RNA extraction and stored at -80°C for analysis.

Clinical assessment

As described earlier (1, 2), the animals were observed on a daily basis and checked for diarrhea, loose stools, bloody stools or any other gross abnormalities. Once the abdominal cavity was opened, observation and evaluation of inflammation was performed according to reported and modified criteria for colonic changes according to parameters like hyperemia, adhesions, ulceration and megacolon (1, 2).

Histological studies

A microscopic assessment of morphological alterations was performed on the descending colon biopsies fixed in 10% buffered formalin and processed according to standard light microscopy techniques.

Serial 5 µm sections were cut and stained with hematoxylin and eosin (H&E) and Periodic Acid Schiff (PAS) using standard methods. The microscopic alterations were checked according to criteria adapted and modified from Hajj Hussein (1), and a numerical score of both colonic changes and histological grade was ascribed by 2 independent researchers (1).

Integrin expression

Was assessed by immunohistochemistry and polymerase chain reaction, depending on the availability of the respective primary antibodies.

Immunohistochemistry for β_2, β_3, and $\alpha_v\beta_3$.

The expression of integrin β_2, β_3, and $\alpha_v\beta_3$ on the paraffin sections from the descending colon was detected using standard immunohistochemistry techniques (1).

Briefly, the 5-µm sections were deparaffinized,

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washed and permeated in 1% bovine serum albumin in phosphate buffered saline solution (PBS, pH=7.6) for 5 min. They were washed with PBS (3x5 min), followed by an overnight incubation period with the primary antibody in a dilution 1/100 (Chemicon, USA and Abcam ab7166-50, USA).

All sections were treated with the secondary antibodies, FITC-Goat anti-mouse IgG, dilution 1/250, (Zymed Labroatories Inc.), for 1.5 h incubation periods. In addition, for control purposes, slides from each group were treated with the secondary antibody only. At the end of the incubation time, tissues were washed with PBS (3x5 min) and antifade reagent was applied. Integrin expression was rated on a scale from 1 to 3 based on fluorescence activity. No activity was scored as 0, low activity was considered as 1, moderate activity as 2, and high activity as 3. This scoring was based on "blind" observations made by 2 independent observers. In addition, tissues were sampled from 6 rats, 5 sections were screened from each rat biopsy per time point, and a score was calculated as an average of 30 readings.

Real-time Quantitative Polymerase Chain Reaction for α_v and β_3

Total RNA extraction from the snap frozen colonic mucosal scrapings, was performed using a Nucleo Spin RNA Total RNA Isolation kit from the Machery-Nagel Company, USA. Real-time PCR was performed on total RNA extracted from the colon mucosa scrapings of the experimental rats using a two-step kit from BioRad Company and according to the supplier instructions.

Total RNA was reverse transcribed into cyclic DNA (cDNA) using the iScript™ cDNA Synthesis Kit from BioRad. Reaction volume per 200 µl-PCR tube was 20 µl, of which 1.5 µl total RNA. The remaining 18.5 µl of the reaction mix were composed of: 13.5 µl of DEPC water, 4 µl of 5X iScript Reaction Mix, and 1 µl of iScript Reverse Transcriptase. Then, the samples were placed in the PCR machine set for reverse transcription, as per the following protocol: 5 min at 25°C, 30 min at 42°C and 5 min at 85°C. Only one cycle of these steps was sufficient to reverse transcribe total RNA.

The obtained cDNA was further amplified using forward and reverse primers for glyceraldehyde-phosphate dehydrogenase (GAPDH), αv integrin and β3 integrin using the iQ™ SYBR® Green Supermix and the iCycler unit, both from BioRad. The primers were obtained from Thermo Fisher Scientific as dried HPLC-pure product. Sterile water was added to each primer tube for a final concentration of 100 pmol/µl (stock solution). Then a working solution for each primer was obtained by the 15:85 dilution of each primer in 85 µl of sterile water. Working solutions were stored at 4°C. The primer nucleotide sequence for each amplified gene was: GAPDH forward 5'-ATGGCACAGTCAAGGCTGAGA-3', reverse 5'-CGCTCCTGGAAGATGGTGAT-3'; α_v integrin forward 5'-CAGTGGCCTTACAAATACAACAACA-3', reverse 5'-TGCAGTTCATTGGTCCATCAA-3'; β_3 integrin forward 5'-CCACTGATGCCAAGACCCATA -3', reverse 5'-TGGCAGCGCCCATCAT-3'. The amplification procedure is detailed as follows. Each 200 µl- PCR tube contained 25 µl reaction solution, 5 µl of which were cDNA template. The remaining 20 µl were composed of: 12.5 µl of iQ SYBR Green Supermix, 5.5 µl of DEPC water, 1 µl of forward primer and 1 µl of reverse primer. A master mix containing the 4 previously cited components was prepared for the required number of samples. 20 µl of the master mix solution were added to each 5 µl-cDNA containing tube. The sample tubes were then ready to be transferred onto the iCycler plate for amplification.

Set point temperature was increased by 0.5°C after cycle 2. Data collection and real-time analysis were enabled also at the end of cycle 2. By the end of cycle 4, melt curve data collection and analysis were enabled. The fluorescent dye used was FAM-490 along with the filter number 2. Cycle threshold (Ct) values were recorded for each sample (in duplicates), reflecting the original RNA concentration for each of the target genes (here α_v and β_3 integrins) compared to the reference gene (GAPDH). The lower the Ct, the more nucleic acid present in the sample. Results were entered, analyzed and plotted on a chart using the Qgene software.

Statistical analysis:

Data were expressed as the means ± SEM for each group of rats. The variation in integrin fluorescence and in each gene level and its corresponding mRNA

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were determined by averaging the values obtained for each group of rats at the indicated time interval. The experimental values were compared to controls and statistical analysis was done using two-way ANOVA and Student's *t*-test to evaluate the difference between individual groups and P value of <0.05 was considered to be statistically significant.

RESULTS

The morphological changes, gross and microscopic, were consistent with previously published data (1, 2). As reported earlier, the descending colon in methylcellulose and bacteria groups, (MC and B), looked normal, while vasodilation, adhesion and necrosis were observed in the iodoacetamide group (IA), and to a much greater extent in the combined IA+B groups at all time points (1, 2).

Altered expression of β_2 integrin in colitis

Data showed an upregulation of β_2 integrins in the inflamed IA and IA+B colonic tissues. The β_2 family of integrins plays a role in normal intestinal homeostasis and can act as a ligand for the cell adhesion molecule ICAM, promoting the extravasation of leukocytes and enhancing the inflammatory reaction. To assess the time course of changes of β_2 expression during colitis, four time points were selected; days 3, 14, 28 and 56.

Immunohistochemistry data showed a 3-4 fold increase in β_2 integrin expression as early as day 3 post-injection in the IA(1.83±41) or IA+B (1.67±0.26) groups, compared to the control groups MC (0.42±3.8) and B (0.67±0.41) (Fig. 1A, B, C, Fig. 2 and Table I). This increased expression was observed in the colon superficial layer among epithelial cells of the mucosa, on leukocytes of

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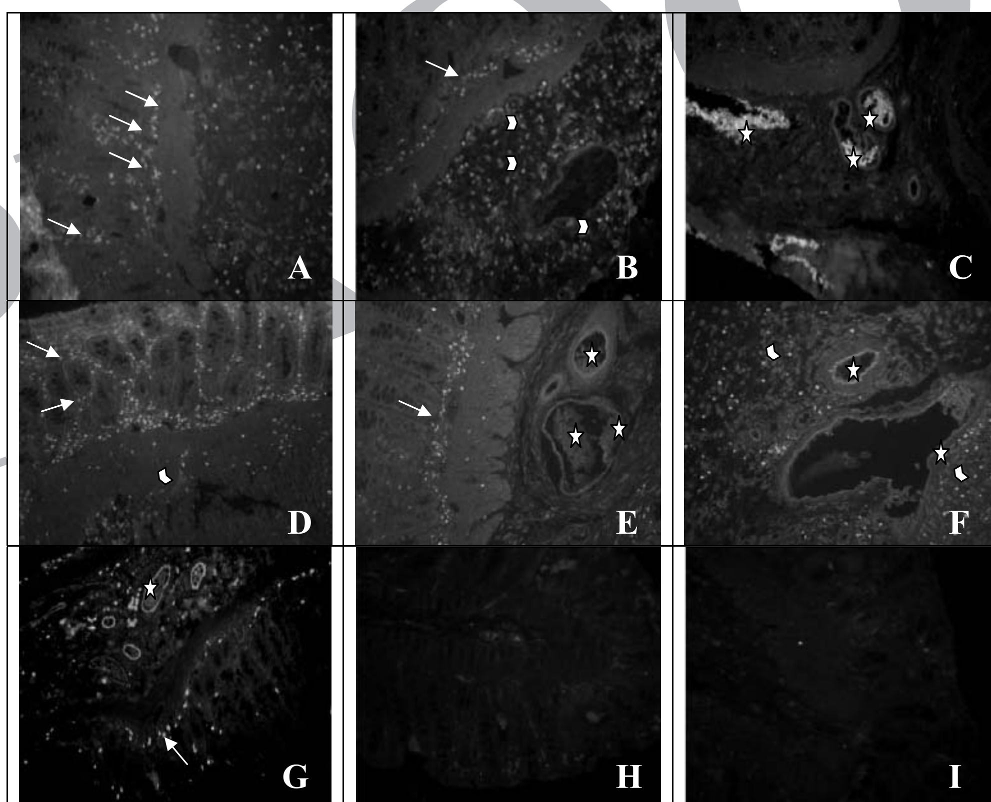

Fig. 1. *Immunodetection of integrins in the descending colon of rats of the combined treatment group (x200).* ***A***, ***B***, *and* ***C*** *show high fluorescence levels of β_2 integrins in the mucosa (arrows), submucosa (arrow heads) and inside, and around blood vessels (stars).* ***D***, ***E***, *and* ***F*** *show high fluorescence levels of β_3 integrins in the mucosa (arrows), submucosa (arrow heads), inside and around blood vessels (stars).* ***G*** *shows the high fluorescence levels of $\alpha_v\beta_3$ integrins again in the mucosa (arrows), submucosa (arrow heads) and around blood vessels (stars).* ***H*** *and* ***I*** *represent controls of methylcellulose and bacteria respectively.*

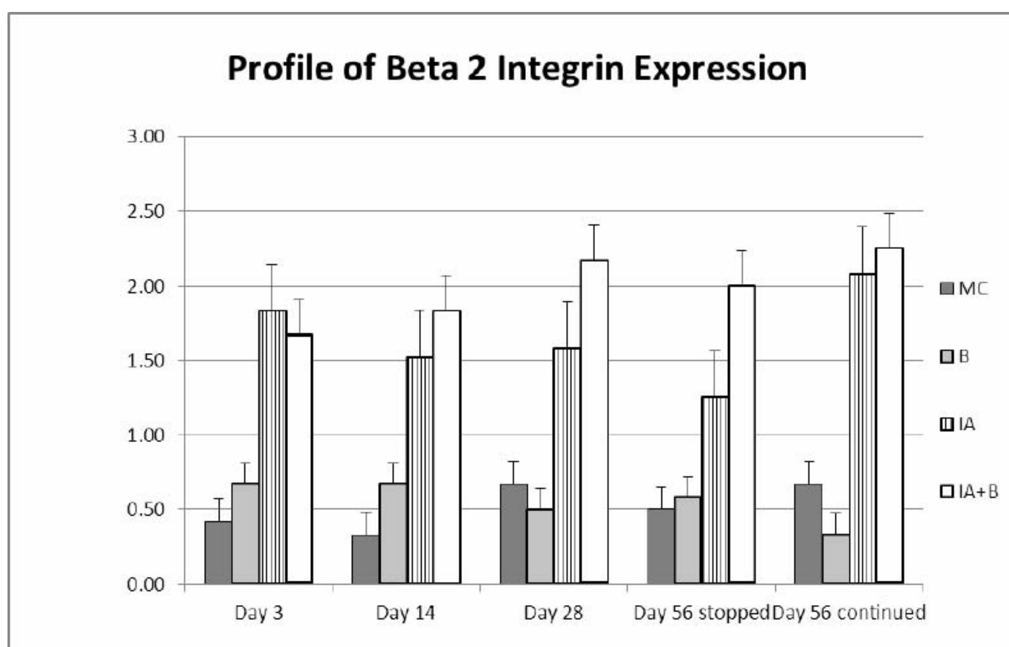

Fig. 2. *Average immunohistochemical scores for β_2 integrin on rat colon (Average±SEM) C and S refer to continuous (**C**) and discontinuous (**S**) intra-rectal inoculation respectively.*

Table I. *Immunofluorescence scores for β_2 integrin (Average ± SEM).*

	MC	B	IA	IA+B
Day 3	0.42±0.38	0.67±0.41	1.83±0.41	1.67±0.26
Day 14	0.33±0.41	0.67±0.41	1.52±0.58	1.83±0.41
Day 28	0.67±0.26	0.50±0.32	1.58±0.38	2.17±0.52
Day 56 stopped	0.50±0.32	0.58±0.38	1.25±0.27	2.00±0.32
Day 56 continued	0.67±0.26	0.33±0.26	2.08±0.74	2.25±0.52

the lamina propria, in the submucosa and inside submucosal blood vessels. β_2 expression remained substantially elevated till day 56 the experimental end-point, even when the induction stopped at day 28 (2.00±0.32) for discontinued and (2.25±0.52) for continued inculcation (Fig. 2 and Table I). It is very important to stress the fact that the blood vessels were engorged with blood cells and the mucosa was heavily infiltrated with leukocytes staining positive for $\beta_{2,}$ during the whole course of the experiment, indicating a sustained inflammatory response (Fig. 1A, B, C). This observation correlated well with a significantly inflamed mucosal histology (1).

Modulation of β_3 integrin expression in colitis:

β_3 integrin family includes two members: $\alpha_v\beta_3$ and $\alpha_{11b}\beta_3$ integrins. The $\alpha_{11b\beta3}$ integrins are found on the surface of platelets and megakaryocytes. The expression of $\alpha_v\beta_3$ integrin is much more ubiquitous in distribution, particularly on endothelial cells, and smooth muscle cells.

The immunohistochemistry studies to determine β_3 integrin expression on the descending colon sections demonstrated variations in expression levels in the different experimental groups. (Fig. 1D, E, F, Fig. 3 and Table II) .Throughout the duration of the experiment, β_3 integrin expression in the MC group was low (Fig. 1H), close to the negative control, 0.08±0.08. The expression was mainly on the endothelial cells ranging from 0.08±0.08 to 0.17±0.11 at all-time points (Table II).

Similarly, the EPEC bacteria group had low levels

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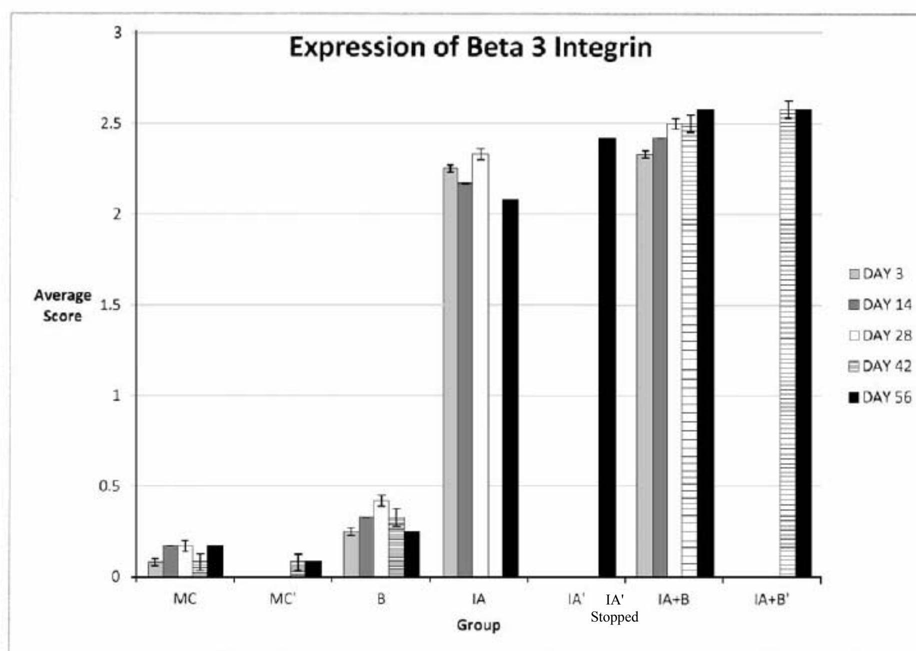

Fig. 3. *Average scores of beta 3 integrin of all groups throughout the experiment as detected by the immunofluorescence. Note the highest levels in IA+B at all time points followed by the IA group. The MC and B groups showed the lowest endothelial expressions at all time points.*

Table II. *IF scores of expression of beta 3 integrin at various time points.*

	MC	B	IA	IA+B
Day 3	0.42±0.38	0.67±0.41	1.83±0.41	1.67±0.26
Day 14	0.33±0.41	0.67±0.41	1.52±0.58	1.83±0.41
Day 28	0.67±0.26	0.50±0.32	1.58±0.38	2.17±0.52
Day 56 stopped	0.50±0.32	0.58±0.38	1.25±0.27	2.00±0.32
Day 56 continued	0.67±0.26	0.33±0.26	2.08±0.74	2.25±0.52

** Groups where inoculations were stopped(s).*

of β_3 integrin expression, (Fig. 1I, Fig. 3 and Table II) and was in the range of 0.25±0.11 to 0.42±0.15 at all-time points in both the vessel wall (endothelium) and vessel content (platelets).

In contrast, the expression pattern of β_3 in the IA group was significantly much higher, in the range of 2.08±0.15 to 2.33±0.11 for continuous inoculation and 2.42±0.15 for the discontinued group on day 56. The β_3 integrin was markedly expressed on endothelial cells in the blood vessels and on platelets. Similar expression was also present in the submucosal layer and extending to the base of the villi of the mucosa layer. In the IA groups (continued and discontinued) β_3 integrin expression was highest in the mucosa, particularly in the lamina propria on day 56, the end of the experiment (Fig. 3 and Table II).

Moreover, the IA+B groups (continued and discontinued) exhibited significantly the highest levels of_β_3 integrin expression both on endothelial cells and platelets, at all time points. The immunohistochemistal analysis of these tissues demonstrated that the highest expression was on the endothelial cells in the mucosa and submucosa with scores ranging from 2.33±0.2 (day 3) to 2.58±0.15 (day 56). The expression of β_3 integrin on platelets was also very high, extending from the submucosal layer of the descending colon to the base of the villi in the mucosa. The continued and discontinued groups of the combined treatment (IA+B) showed similar β_3 expressions (Fig. 1D, E, F, Fig. 3 and Table II).

In addition, the expression of the β_3 integrin

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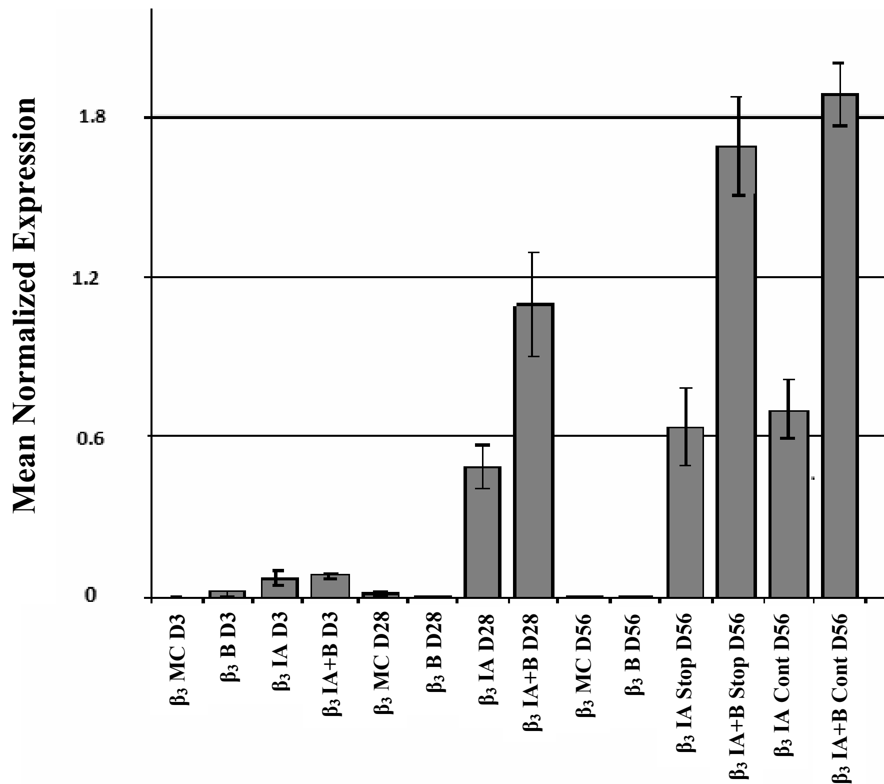

Fig. 4. *Expression of Beta 3 Integrin Gene of all Groups at days 3, 28, and 56. Note the minimal expression alone broad line controls on day 3, the significant expression in group inoculated with IA alone and very significant expression on days 28 and 56 for the IA+B groups.*

Table III. *IF scores for $\alpha_v\beta_3$ integrin staining.*

	MC	B	IA	IA+B
Day 3	0.42±0.38	0.25±0.27	1.08±0.58	0.75±0.61
Day 14	0.33±0.26	0.17±0.26	1.67±0.26	1.83±0.41
Day 28	0.33±0.26	0.75±0.27	1.75±0.27	1.75±0.27
Day 56 stopped	0.50±0.32	0.58±0.20	1.42±0.20	2.00±0.65
Day 56 continued	0.58±0.20	0.58±0.20	1.75±0.27	2.00±0.32

gene, as determined by real time PCR, showed as expected, the lowest levels in the MC control and bacteria groups at the three tested time points, days 3, 28, and 56, see (Fig. 4 and Table III). However, at these three time points, the combined iodoacetamide and bacteria groups (IA+B) had the highest levels of expression to (day 28), 1.6 and 1.82 (day 56) for discontinued and continued inoculation groups, respectively (Fig. 4 and Table III). A similar trend but to much lesser extent was observed in the IA group alone 0.5 (day 28), and 0.7 and 0.8 on day 56 for discontinued and continued inculcation respectively followed by the iodoacetamide treated groups. At day 28, the combined treatment group showed a relatively higher expression of β_3 gene. At day 56, the expression of β_3 integrin in the continued and discontinued combined treatment groups was also higher than in the iodoacetamide groups. The highest level of expression of β_3 integrin was on day 56 for both the continued and discontinued combined treatment groups (Fig. 4 and Table III).

In summary, β_3 expression was the highest in the IA+B group. It was mostly expressed on the endothelial cells and platelets. Compared to the

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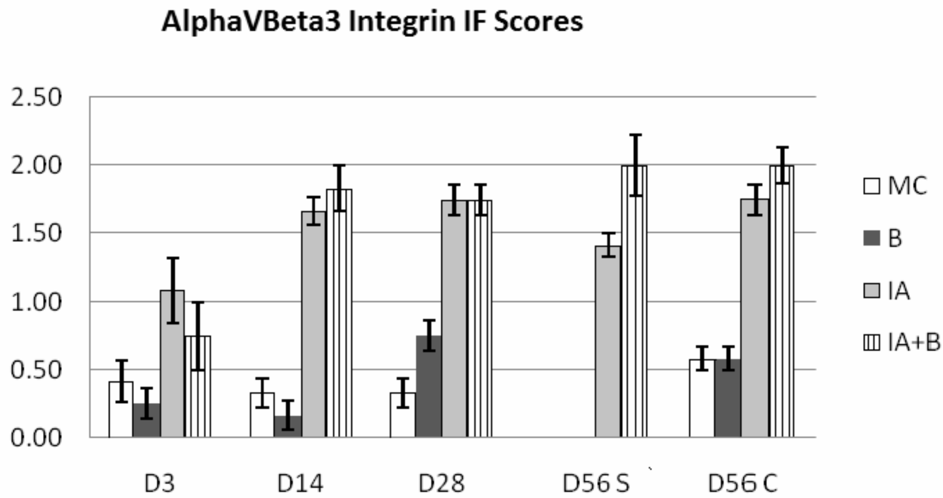


Fig. 5. *Average scores for $\alpha_v\beta_3$ integrin on rat colon. Note the continous increase of $\alpha_v\beta_3$ expression over time in the IA+B groups.*

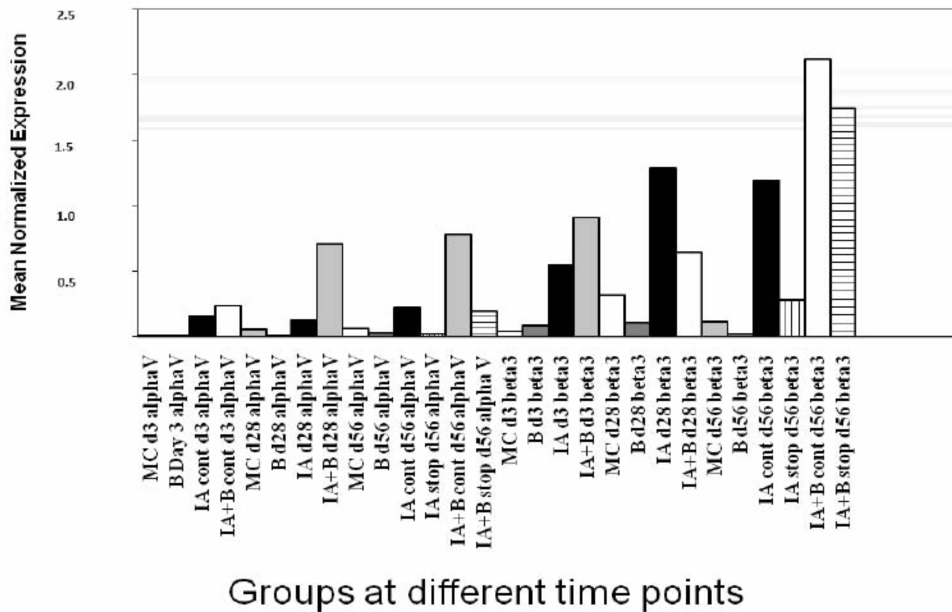


Fig. 6. *Gene expression of αV and β3 integrin subunits on days 3, 28 and 56. Note the continuous increase in the IA+B groups.*

control MC and B, the group inoculated with IA alone did show a significant increase but not to the same extent as the combined treatment groups. The presence of EPEC, in addition to IA, significantly increased the expression of β_3 integrin, on leukocytes, platelets, and endothelial cells, throughout the duration of the experiment (Fig. 4 and Table III).

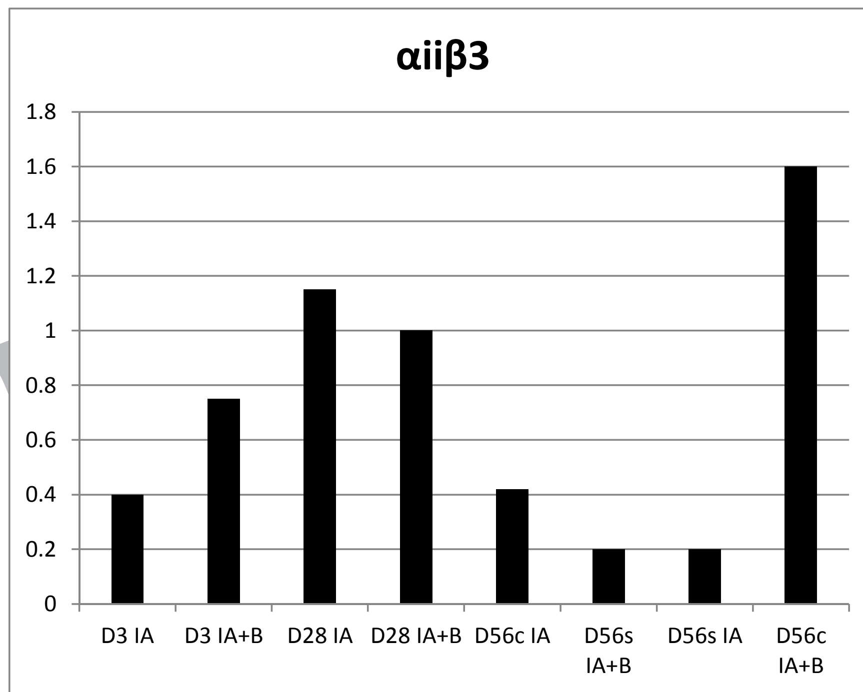
Alteration of $\alpha_v\beta_3$ and αiiβ3 integrin expression

Expression of the $\alpha_v\beta_3$ integrin complex was

Table IV. *Expression scores for the various integrins.*

Time	Category	β3	αvβ3	αiiβ3
D3	IA	0.6	0.2	0.4
D3	IA+B	1.0	0.25	0.75
D28	IA	1.3	0.15	1.15
D28	IA+B	1.75	0.75	1.0
D56s	IA	1.25	0.8	0.42
D56s	IA+B	2.0	1.8	0.2
D56c	IA	0.35	0.1	0.25
D56c	IA+B	1.8	0.2	1.6

Fig. 7. *Expression of $\alpha ii\beta_3$. Note the persistent increase with the IA+B treatment till endpoint of 56 days.*



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assessed by both immunohistochemistry and polymerase chain reaction. It is usually expressed on activated endothelial cells and smooth muscle cells among others, and its common ligands are vitronectin, fibrinogen and osteopontin. An increase in expression could indicate a vascular response to injury, neointimal formation, leukocyte trafficking, and vascular cell apoptosis. The immunohistochemical analysis, using a monoclonal primary antibody against the $\alpha_v\beta_3$ integrin complex, demonstrated low expression in the control group of MC ranging, during the all duration of 56 days, from a fluorescence score of 0.33±0.26 to 0.58±0.2 on a scale of 3 with an overall average of 0.42±0.11. Similar results were obtained from the B group, with a fluorescence score of 0.17±0.26 to 0.75±0.27. However, a significantly higher expression was present in the IA group with continued treatment, fluorescence scores ranging from 1.08±0.58 (day 3) to 1.75±0.27 (day 56), or discontinued treatment, fluorescence scores ranging from 1.08±0.27 (day 3) to 1.42±0.2 (day 56). In the combined treatment groups, expression of the $\alpha_v\beta_3$ integrin complex was highest with a fluorescence score increasing steadily from 0.75±0.6 (day 3) to 2.0±0.65 (day 56). Expression remained high throughout the duration of

the experiment. It was detected in almost all layers, but more so in the mucosa and submucosa, in and around the blood vessels (Fig. 1G, Fig. 5 and Table III).

In addition, analysis of data from RT-RCR showed that at the selected time points, days 3, 28 and 58, the expression of the α_v gene was lowest in the control group of MC and EPEC, followed by the IA group (Fig. 6). On the other hand, the group receiving the combined IA+EPEC treatment throughout the study showed the highest expression of α_v integrin, which increased over time. In contrast, in the IA+B discontinued treatment group, the α_v gene was expressed at much lower levels than the IA+B continued treatment group, but slightly higher than the control levels (Fig. 6).

In summary, significant upregulation of β_3 or $\alpha_v\beta_3$ expression occurred in the colonic colitis tissues compared to controls. Overall, the RT-PCR results were concordant with the expression levels detected by immunohistochemistry.

Quantitative extrapolation of the $\alpha ii\beta_3$ expression

Additional analysis of the αvβ3 and β_3 expression data was performed taking into consideration that β_3 could be associated to either α_v or $\alpha ii\beta_3$ in the inflamed colon.

As shown in Table IV, the expression of αiiβ3 increased on days 3 and 28 and kept increasing till day 56; in the continuous from 0.75 (day 3) to 1.0 (day 28) and 1.6 (day 56). The same treatment group of IA+B. However, with IA alone the levels oscillated slightly up and down with fluorescence scores between 0.4 (day 3) 0.15 (day 28), and .25 (day 56), (Fig.7 and Table IV).

As is evident from Table IV, the αiiβ3, resulting from the calculated difference between β_3 and αiiβ3 increased to almost double in the presence of bacteria, on day 3 from 0.4 to 0.75. It kept increasing till day 56 to peak at 1.6 in IA+B compared to 0.25 in IA. The highest level of αiiβ3 was obtained with the continuous presence of B and IA.

DISCUSSION

This study reconfirmed the involvement of bacteria in exacerbating and maintaining an inflammatory process, induced by a chemical (iodoacetamide) and bacteria (enteropathogenic *E. coli*) administered directly into the intestinal lumen (1-3). The enteropathogenic *E. coli* used is invasive, has both attaching and effacing capabilities and could penetrate epithelial cells, especially in the inflammatory environment induced by the chemical insult, altering cell adhesion molecules including the various integrins and facilitating the infiltration of cells (21, 22).

The methylcellulose-treated rats as well as the bacteria-inoculated ones had no signs of illness with normal abdominal viscera upon inspection. The iodoacetamide-injected animals, however, showed significantly higher scores of macroscopic alterations when compared to the MC and to the bacteria-inoculated group. On the other hand, the combined-induction group, showed the highest macroscopic alterations scores among all experimental groups, leading, along with IA, to upregulation of the β_2, $\alpha ii\beta_3$ and $\alpha_v\beta_3$ integrins among others (23). The aggravating effect of the EPEC on inflamed tissues could be explained by the fact that damaged mucosa impairs the intestinal barrier function, allowing the invasion of the underlying intestinal layers by the bacterium itself or by bacterial toxins of the same or other strains of bacteria.

Data showed that $\alpha_v\beta_3$ integrins levels in the colonic mucosa were high in the IA and the IA+B groups. The high expression of $\alpha_V\beta_3$ integrins, especially on starting day 28 of the experiment, could be explained by the appearance of newly forming endothelial and smooth muscle cells, which could occur in parallel with malignancies of the intestine (8-12, 16-18). This is very likely to occur in IBD, considering the normally high epithelial turnover of the intestines, further enhanced by tissue damage and the emergence of tissue repair by rapid multiplication of the cells present. Mutations and mitotic defects might occur and lead to the development of cellular masses that require blood supply. Increased levels of $\alpha_v\beta_3$ integrins in the mucosa, could be associated with the onset of early tumor growth, or at least pathological polyps and masses following continued inflammation (5, 23-25). In fact, on days 42 and 56 of the experiment, the highest level of $\alpha_v\beta_3$ integrin expression on blood vessels and other immune and endothelial cells, was depicted (8).

In addition, data revealed increased expression

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of $\alpha_{IIb}\beta_3$ integrin on platelets in both IA and IA+B groups, perhaps indicative of increased platelet activation (26). Along with $\alpha_{IIb}\beta_3$ integrin activation these activated platelets release pro-inflammatory mediators and induce inflammatory cell chemotaxis. Such increase in $\alpha_{IIb}\beta_3$ integrin expression could also be correlated to platelet aggregation for thrombus formation in cases of vascular injuries (14, 26). Both the IA and IA + B groups exhibited elevated expression of $\alpha_{IIb}\beta_3$ integrin on the platelets, reflecting the high inflammatory and thrombotic functions of these platelets (10, 13). Another integrin molecule of similar importance, the β_2 integrin, normally, expressed on all subtypes of white blood cells, from both the myeloid and lymphoid lineages and their expression is enhanced in Crohn's and ulcerative colitis (8). The IA and IA+B groups showed extensive β_2 integrin expression indicating more abundant infiltration of immune cells. A close examination of the results for β_2 integrin showed that the descending colon of the combined-induction group of animals expressed higher levels of β_2 integrins than the exclusively chemically challenged group, proving that the addition of a bacterial (EPEC) challenge to the chemical offense exacerbated tissue damage, in concordance with the aggravated clinical symptoms and histopathological features.

However, the surge in the β2 integrin on days 28 and 42, followed by the corresponding increase in α vβ3 integrins detected on days 42 and 56 was not probably sufficient for the onset of a malignant tumor, for many other factors interfere to direct the cellular mass into either a benign mass or a malignant growth. However, the surge in $\alpha_v\beta_3$ integrin expression indicated an abnormal cellular expansion that is likely to turn cancerous (16, 28). This explains the relevance of assessing the expression of $\alpha_v\beta_3$ integrins in order to check for possible tumor onset following long-standing inflammation (11, 26). In brief, the high expression levels of the various integrins were consistent with the high inflammatory cell infiltrates observed in the histological sections, as well as with the sick clinical profile of the challenged rats (3, 21). This will be the focus of future research.

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