CD4⁺CD45RB⁺ T cell transfer induced colitis in mice is accompanied by osteopenia which is treatable with recombinant human osteoprotegerin


Background and aims: Transfer of CD4⁺CD45RB⁺ T cells into semi syngeneic immunodeficient mice represents a model of inflammatory bowel disease (IBD). As patients with IBD often suffer from osteopenia, we studied if this T cell transfer in mice results in osteopenia in addition to colitis, and if treatment with osteoprotegerin (OPG) has effects on the bone mineral density of T cell transferred mice. We also investigated whether osteopenia was due to malabsorption as a result of a dysregulated digestive tract or as a consequence of the inflammatory process.

Methods: CD4⁺CD45RB⁺ or CD4⁺CD45RB⁺ T cells (4 x 10⁵) were sorted from CB6F1 and transferred into C.B.17 scid/scid mice. Recipient mice were treated with human IgG1 Fc (control) or Fc-OPG three times per week in a prophylactic regimen as well as a therapeutic regimen (after 10% body weight loss) and were evaluated for osteopenia and colitis.

Results: Mice that received CD4⁺CD45RB⁺ T cells developed osteopenia (as indicated by decreased bone density accompanied by increased osteoblasts and increased osteoclasts) and colitis (as indicated by histological changes in the large intestine). Mice that received CD4⁺CD45RB⁺ T cells developed neither osteopenia nor colitis. All animals consumed, on average, the same amount of food and water over the course of the study. Prophylactic treatment of Fc-OPG increased bone density in mice that received either CD4⁺CD45RB⁺ or CD4⁺CD45RB⁺ T cells but had no effects on the gastrointestinal tract. Fc-OPG treatment of osteopenic mice with established IBD caused the normalisation of bone density. Osteopenia in CD4⁺CD45RB⁺ T cell recipients was accompanied by hypoparathyroidism that was partially normalised by treatment with Fc-OPG. CD4⁺CD45RB⁺ T cell recipients also had a bone marrow inflammatory cell infiltrate expressing tumour necrosis factor α which was unaffected by treatment with Fc-OPG.

Conclusions: CD4⁺CD45RB⁺ T cell transfer results in osteopenia in addition to colitis. Evidence suggests that this osteopenia was induced by inflammatory cell infiltration and not by malabsorption of calcium. Recombinant human osteoprotegerin effectively treated the osteopenia. OPG may be a useful therapeutic option for treating osteopenia in patients with IBD.

Materials and methods
CB6F1 mice aged 12–14 weeks from Charles River Laboratories (Worcester, Massachusetts, USA) were used as T cell donors while female C.B.17 scid/scid mice aged 14–16 weeks were used as recipients (Jackson Laboratories Bar Harbour, Maine, USA). The fusion protein IgG1Fc-hOPG was prepared as previously described. OPG would prevent or reverse any bone loss in this disease model. Finally, we hoped to assess the relative contributions of inflammation and/or malabsorption to any observed bone loss.
from CB6F1 mice into immunodeficient C.B17 scid/scid mice. Mice were injected subcutaneously with 5.0 mg/kg of Fc-OPG or 3.4 mg/kg of Fc (the equimolar amount) thrice weekly starting on day 0 after disease induction. A second group of mice (CD4+CD45RBHi) were started on Fc-OPG treatment after 10% body weight loss. Mice were housed individually with food and water depletion monitored on a weekly basis and were sacrificed on day 34. Experiments were performed on groups of 4–10 mice and repeated at least once.

Necropsy
Mice were euthanised by CO2 asphyxiation and blood taken for complete blood counts and to derive serum. Tissue samples were fixed in formalin and later stained with haematoxylin and eosin. 25 Tibias and femurs were also isolated and assayed using peripheral quantitative computer tomography (pQCT) and radiography or prepared for histological examination according to standard procedures. The thoracic vertebrae, as one unit, were fixed and prepared for histological examination as above. The lumbar vertebrae were removed and stored in 70% ethanol: L5 was recognised as the first vertebra next to the iliac crest and subjected to pQCT.

Radiography and bone mineral density measurement
Femurs fixed in 70% ethanol were radiographed with a 43855A x-ray system (Faxitron X-ray, Buffalo Grove, Illinois, USA) using exposure times of 49 seconds, and magnified with DeskScan II scanning software (Hewlett Packard, Palo Alto, California, USA). BMD was evaluated by pQCT using tibias stored in 70% ethanol and an XCT-960M scanner (Norland Medical Systems, Ft Atkinson, Wisconsin, USA) and related XMICE software (Stratec, Frankfurt, Germany). Two 1.25 mm cross sections of the proximal metaphysis of the tibia, 1.5 mm and 2 mm, respectively, from the end of the bone, were analysed to determine total and trabecular BMD and their average value calculated. To determine cortical BMD, a single 1.25 mm cross section of the diaphysis of the tibia, 4 mm from the proximal end of the bone, was analysed. Total BMD was also determined analysing the lumbar vertebra L5 and focusing on a single 1.25 mm mid-vertebral cross section.

Bone histomorphometry
Histomorphometric analysis was performed using paraffin embedded sections of the tibias, as previously described. Osteoblasts were identified morphologically from sections stained with haematoxylin and osteoclasts by staining for cathepsin K and haematoxylin. Osteoblast and osteoclast perimeter was determined as the perimeter of cells in direct contact with cancellous bone surfaces.

Histological examination of the large intestine and bone
Histopathological damage of the proximal, middle, and distal large intestine was semi quantitatively scored as a global assessment of inflammation on a scale of 0–5. Histopathological damage of the bone was assessed by examining tibias and thoracic vertebrae stained with haematoxylin and eosin. Damage was scored semi quantitatively using the aforementioned scoring system, based...
primarily on inflammatory infiltrate. All samples were scored by a pathologist in a blinded fashion.

Serum amyloid A, complete blood counts, and clinical chemistries

Serum amyloid A (SAA) was measured in serum by enzyme linked immunosorbent assay using a commercially available kit (BioSource International, Camarillo, California, USA). Complete blood counts were obtained using a H1E counter (Technicon, Tarrytown, New York, USA). Serum was analysed for the presence of calcium, phosphorous, and alkaline phosphatase activity using specific diagnostic kits from Roche Diagnostics (New Jersey, USA) on a Hitachi 717 chemistry analyser (Hitachi, New Jersey, USA). Serum tartrate resistant acid phosphatase (TRAP) was analysed on the same machine using kit reagents from Sigma Diagnostics (St Louis, Missouri, USA). Parathyroid hormone (PTH) was measured in serum by radioimmunoassay using a commercially available kit (Immutopics, San Clemente, California, USA).

In situ hybridisation

An antisense RNA probe for a fragment of the murine tumour necrosis factor α (TNF-α nucleotides 429–595 of GenBank accession No M13049.1) was synthesised from a linearised plasmid template with [33P]UTP (Amersham, Piscataway, New Jersey, USA) and the Sp6 RNA polymerase (Ambion, Austin, Texas, USA). Sections (5 μM) of the paraffin embedded, formalin fixed, decalcified tibia were processed by standard in situ hybridisation techniques, as previously described, and hybridised overnight with 2 × 10⁶ counts per minute of probe. TNF-α mRNA expression in the tibia and femur was designated positive or negative, indicating the intensity and extent of signal in the entire section. All samples were scored in a blinded fashion.

Statistical analysis

Results are given as means (SEM) of the data set. Body weight curves were compared using the t statistic in an ANOVA with Dunnett’s correction. Continuous variables such as BMD, and discrete variables such as histopathological scores, were compared using the unpaired heteroschedastic Student’s t test. Variables that were scored as positive or negative (such as presence or absence of TNF-α) were analysed by χ² analysis.

RESULTS

Transfer of CD4⁺CD45RBlow T cells to immunodeficient mice caused a significant decrease in the radio-opacity of the distal femur (B) compared with transfer of CD4⁺CD45RBhigh T cells (A). Prophylactic administration of Fc-osteoprotegerin (OPG) to the CD4⁺CD45RBhigh mice noticeably increased the radio-opacity of the distal femur (C). Prophylactic Fc-OPG also restored the radio-opacity of the distal femur of diseased CD4⁺CD45RBlow mice (D). Disease was induced by injection of 4 × 10⁶ sorted CD4⁺CD45RBlow T cells into scid/scid mice. Negative control mice received 4 × 10⁵ sorted CD4⁺CD45RBlow T cells. Treatment consisted of subcutaneous injection of 5.0 mg/kg of Fc-OPG or 3.4 mg/kg of Fc control three times per week starting on day 0. For the therapeutic group, treatment with Fc-OPG started when individual animals had attained 10% body weight loss. Treatment was continued in all cases until necropsy on day 34. Haematoxylin and eosin stained biopsies of the proximal, middle, and distal large intestine were assessed for extent of inflammation on a scale of 0–5 (0 = absent, 5 = severe) by a pathologist in a blinded fashion and then averaged. In all cases results represent mean (SEM) values, with n = 7–8 for the CD4⁺CD45RBlow groups and n = 4 for the CD4⁺CD45RBhigh groups. Statistical significance of the data was determined using the unpaired heteroschedastic Student’s t test. **p < 0.001.
Treatment of IBD related osteopenia in mice

Disease was induced by injection of 4×10⁵ sorted CD4+CD45RB⁺ T cells into scid/scid mice. Negative control mice received 4×10⁵ CD4+CD45RBlow cells. Treatment consisted of subcutaneous injection of 5.0 mg/kg of Fc-OPG or 3.4 mg/kg of Fc control three times per week starting on day 0. For the therapeutic group, treatment with Fc-OPG started when individual animals had attained 10% body weight loss. Treatment was continued in all cases until necropsy on day 34. Results represent mean (SEM) values, with n = 7–8 for the CD4+CD45RBlow groups and n = 4 for the CD4+CD45RBhi groups. Statistical significance versus the CD4+CD45RBlow group treated with Fc was calculated by the probability associated with the unpaired heteroscedastic Student's t test: *p<0.05, ***p<0.001.

Figure 4 Transfer of CD4+CD45RBhi T cells (RB Hi) to scid/scid mice caused a highly significant decrease in total bone mineral density (BMD) of the tibia compared with transfer of CD4+CD45RBlow T cells (RB Lo) (A). CD4+CD45RBlow T cells also caused a decrease in total BMD of the L5 lumbar vertebra (B). Administration of Fc-osteoprotegerin (OPG) to CD4+CD45RBhi mice supranormalised total BMD of the tibia but had no significant effect on total BMD of the lumbar vertebra. Prophylactic Fc-OPG also supranormalised total BMD of the tibia of diseased CD4+CD45RBhi mice and therapeutic administration normalised it. Fc-OPG had no significant effect on total BMD of the L5 lumbar vertebra. Disease was induced by injection of 4×10⁵ sorted CD4+CD45RBlow T cells into scid/scid mice. Negative control mice received 4×10⁵ CD4+CD45RBlow cells. Treatment consisted of subcutaneous injection of 5.0 mg/kg of Fc-OPG or 3.4 mg/kg of Fc control three times per week starting on day 0. For the therapeutic group, treatment with Fc-OPG started when individual animals had attained 10% body weight loss. Treatment was continued in all cases until necropsy on day 34. Results represent mean (SEM) values, with n = 7–8 for the CD4+CD45RBlow groups and n = 4 for the CD4+CD45RBhi groups. Statistical significance versus the CD4+CD45RBlow group treated with Fc was calculated by the probability associated with the unpaired heteroscedastic Student's t test: *p<0.05, ***p<0.001.

Figure 5 Transfer of CD4+CD45RBhi T cells (RB Hi) to scid/scid mice caused a significant decrease in the number of osteoblasts (A) and an increase in the number of osteoclasts (B) in the tibia-femur compared with transfer of CD4+CD45RBlow T cells (RB Lo). Administration of Fc-osteoprotegerin (OPG) to control CD4+CD45RBhi mice completely eliminated both osteoblasts and osteoclasts. Fc-OPG, administered either prophylactically or therapeutically, also eliminated all osteoblasts and osteoclasts from the tibia-femur of diseased CD4+CD45RBhi mice. Disease was induced by injection of 4×10⁵ sorted CD4+CD45RBlow T cells into scid/scid mice. Negative control mice received 4×10⁵ CD4+CD45RBlow cells. Treatment consisted of subcutaneous injection of 5.0 mg/kg of Fc-OPG or 3.4 mg/kg of Fc control three times per week starting on day 0. For the therapeutic group, treatment with Fc-OPG started when individual animals had attained 10% body weight loss. Treatment was continued in all cases until necropsy on day 34. Results represent mean (SEM) values, with n = 7–8 for the CD4+CD45RBlow groups and n = 4 for the CD4+CD45RBhi groups. The numbers of osteoclasts and osteoblasts were determined by bone histomorphometry, as described in the materials and methods. Statistical significance versus the CD4+CD45RBlow group treated with Fc was calculated by the probability associated with the unpaired heteroscedastic Student's t test: *p<0.05.

CD4+CD45RBhi T cells cause bone density loss, which is prevented or normalised by treatment with Fc-OPG

Standard x rays were taken to visualise any significant alterations in the structure or radio-opacity of the bone. Figure 3 shows that transfer of CD4+CD45RBhi T cells to immunodeficient mice caused a visible decrease in the radio-opacity
of the distal femur (fig 3B) compared with that observed with transfer of CD4⁺CD45RB⁺ T cells (fig 3A). Prophylactic administration of Fc-OPG to control CD4⁺CD45RB⁻ mice visibly increased the radio-opacity of the distal femur (fig 3C). Prophylactic Fc-OPG seemed to normalise the radiographic appearance of the distal femur of diseased CD4⁺CD45RB⁺ mice (fig 3D). We quantified changes in BMD via pQCT analysis of the tibia. Figure 4 demonstrates that transfer of CD4⁺CD45RB⁺ T cells to scid/scid mice caused a highly significant decrease in the total (trabecular plus cortical) BMD of the proximal tibial metaphysis compared with transfer of CD4⁺CD45RB⁻ T cells (p < 0.0001) (fig 4A). CD4⁺CD45RB⁺ T cells also caused a decrease in total BMD of the L5 lumbar vertebra (fig 4B). Administration of Fc-OPG to control CD4⁺CD45RB⁺ mice supranormalised total BMD of the tibia (p = 0.0013) but had no significant effect on total BMD of the L5 lumbar vertebra (p = 0.7846). Prophylactic Fc-OPG also supranormalised BMD of the tibia of diseased CD4⁺CD45RB⁺ mice (p = 0.0003) and therapeutic administration normalised BMD (p = 0.9594). Fc-OPG had no significant effect on BMD of the L5 lumbar vertebra. In all cases, BMD loss in diseased mice was more pronounced in trabecular bone relative to cortical bone (data not shown). In a separate experiment, tibial BMD loss at the 10% body weight loss point (the point of intervention with Fc-OPG) was found to be similar to that at the time of necropsy (p = 0.3052). For reference, previous experiments have shown that there is no significant difference between total, trabecular, and cortical BMD of CD4⁺CD45RB⁻ and normal unreconstituted scid/scid mice. These data clearly show that administration of Fc-OPG significantly increases BMD of normal mice and can both prevent disease associated bone loss as well as normalising loss that has already occurred.

CD4⁺CD45RB⁺ T cells decrease osteoblasts and increase osteoclasts, while Fc-OPG eliminates both cell types

Previous studies with OPG have demonstrated its ability to reduce the numbers of osteoclasts in both normal and inflamed bone. Figure 5 shows that the transfer of CD4⁺CD45RB⁻ T cells to immunodeficient mice caused a significant decrease in the number of osteoblasts (p = 0.0236) (fig 5A) and an increase in the number of osteoclasts (p = 0.0387) (fig 5B) in the tibia compared with transfer of CD4⁺CD45RB⁺ T cells. Administration of Fc-OPG to control CD4⁺CD45RB⁺ mice virtually eliminated both osteoblasts and osteoclasts. Fc-OPG, administered either prophylactically or therapeutically, also eliminated all osteoblasts and osteoclasts from the tibia-femur of diseased CD4⁺CD45RB⁺ mice. This observation is consistent with previous studies, and the simultaneous elimination of osteoblasts is thought to result from the normal coupling of osteoblast and osteoclast metabolism.

CD4⁺CD45RB⁺ T cells decrease circulating parathyroid hormone, which is partially normalised by treatment with Fc-OPG

Disease related malabsorption may include impairment of calcium absorption, which could lead to hypocalcaemia and a
individualised to each mouse) and a similar pattern was observed with the exception that the diseased Fc-OPG therapeutically treated mice had serum PTH levels that were not significantly different from CD4+CD45RBLo control mice \( (p = 0.6406) \) (data not shown).

CD4+CD45RBHi T cells do not affect serum calcium or phosphorous but they increase serum TRAP and decrease serum alkaline phosphatase; Fc-OPG normalises TRAP and further decreases alkaline phosphatase

Immunodeficient mice injected with CD4+CD45RBHi cells had similar levels of serum calcium to mice injected with CD4+CD45RBLo cells \( (p = 0.5666) \), irrespective of treatment with Fc or Fc-OPG (all within the normal range of 8.25–8.50 g/dl (Amgen Inc., historical data)).

Similarly, CD4+CD45RBHi cells did not cause a change in levels of serum phosphorous \( (9.2 (0.4) \text{ g/dl}) \) compared with control CD4+CD45RBLo cells \( (9.0 (0.5) \text{ g/dl}) \; p = 0.7475) \). Diseased CD4+CD45RBHi mice treated prophylactically with Fc-OPG had significantly lower serum phosphorus \( (8.0 (0.4) \text{ g/dl}) \; p = 0.0561) \), as did diseased CD4+CD45RBHi mice treated with therapeutic Fc-OPG \( (7.6 (0.5) \text{ g/dl}) \; p = 0.0421) \). P levels in these two Fc-OPG treatment groups were not significantly different from each other \( (p = 0.6984) \).

CD4+CD45RBHi cells caused a significant rise in levels of serum TRAP \( (6.00 (0.45) \text{ U/l}) \) compared with transfer of CD4+CD45RBLo cells \( (3.48 (0.3) \text{ U/l}) \; p < 0.0001) \). CD4+CD45RBHi mice treated prophylactically with Fc-OPG did not have significantly changed levels of TRAP \( (5.18 (2.13) \text{ U/l}) \; p = 0.4267) \). Levels of TRAP in diseased mice treated prophylactically with Fc-OPG \( (4.24 (0.85) \text{ U/l}) \) were normalised and were not significantly different to CD4+CD45RBLo normal controls treated with Fc \( (p = 0.3854) \). TRAP levels in diseased mice treated with therapeutic Fc-OPG were also normalised \( (4.48 (0.27) \text{ U/l}) \) but were still significantly higher than normal controls treated with Fc \( (p = 0.0133) \). This observation is consistent with the known correlation of TRAP levels with osteoclast numbers.20

CD4+CD45RBHi mice had significantly lower levels of alkaline phosphatase (ALP) \( (54.9 (3.5) \text{ U/l}) \) compared with control CD4+CD45RBLo mice \( (83.3 (3.8) \text{ U/l}) \; p < 0.0001) \). Control mice treated with Fc-OPG also had depressed levels of ALP \( (52.3 (3.6) \text{ U/l}) \; p < 0.0001) \). Levels of ALP in diseased mice treated with prophylactic Fc-OPG were depressed even further \( (34.6 (1.3) \text{ U/l}) ; p = 0.0002 \) relative to control CD4+CD45RBLo mice), and similarly in therapeutic Fc-OPG treatment \( (40.6 (4.6) \text{ U/l}) \; p = 0.0002 \) relative to control CD4+CD45RBLo mice. Serum ALP levels in the two diseased Fc-OPG treatment groups were not significantly different from each other \( (p = 0.1570) \). This change in ALP levels is consistent with the known correlation of ALP with osteoblast numbers and is consistent with the histomorphometric analysis. Levels of ALP fall in diseased mice as the numbers of osteoblasts decreases and treatment of diseased mice with Fc-OPG eliminates both osteoclasts and osteoblasts, further lowering the levels of ALP.20 21

CD4+CD45RBHi T cells cause a TNF-α expressing bone inflammatory infiltrate, which is unaffected by treatment with Fc-OPG

Previous studies with OPG in inflammatory models of disease have established that OPG exerts its effect by specifically targeting osteoclasts that mediate bone resorption.20 21 Hence we examined whether there was significant inflammatory cell infiltrate in the bones of diseased mice in this model of IBD. Transfer of CD4+CD45RBHi T cells to scid/scid mice induced an inflammatory cell infiltrate (fig 6B, main picture and inset) and an increase in the numbers of osteoclasts.
...materials and methods, and scoring was performed by a pathologist in a blinded fashion for the presence or absence of signal.

Treatment consisted of subcutaneous injection of 5.0 mg/kg of Fc-OPG or 3.4 mg/kg of Fc control three times per week starting on day 0. Treatment was continued in all cases until necropsy on day 34. In situ hybridisation using a standard murine TNF-α a probe was performed, as described in the materials and methods, and scoring was performed by a pathologist in a blinded fashion for the presence or absence of signal.

Figure 8 Transfer of CD4+CD45RBhi T cells to scid/scid mice caused significant upregulation of tumour necrosis factor α (TNF-α) mRNA in the shaft of the tibia (B) compared with transfer of CD4+CD45RBlo T cells, which had no significant effect on expression of TNF-α mRNA (A). Administration of Fc-osteoprotegerin (OPG) had no significant effect on expression of TNF-α mRNA in the shaft of the tibia/femur in either CD4+CD45RBhi mice (C) or CD4+CD45RBlo mice (D). (A) CD4+CD45RBlo cells treated with 3.4 mg/kg of Fc three times per week starting on day 0. (B) CD4+CD45RBhi cells treated with 3.4 mg/kg of Fc three times per week starting on day 0. (C) CD4+CD45RBlo cells treated with 5.0 mg/kg of Fc-OPG control three times per week starting on day 0. (D) CD4+CD45RBhi cells treated with 5.0 mg/kg of Fc-OPG three times per week starting on day 0. Disease was induced by injection of $4 \times 10^5$ sorted CD4+CD45RBhi T cells into scid/scid mice. Negative control mice received $4 \times 10^5$ non-pathogenic CD4+CD45RBlo cells. Treatment consisted of subcutaneous injection of 5.0 mg/kg of Fc-OPG or 3.4 mg/kg of Fc control three times per week starting on day 0. Treatment was continued in all cases until necropsy on day 34. In situ hybridisation using a standard murine TNF-α probe was performed, as described in the materials and methods, and scoring was performed by a pathologist in a blinded fashion for the presence or absence of signal.

DISCUSSION

We have shown that induction of a Crohn’s disease like phenotype in mice results in significant loss of BMD. Treatment with recombinant OPG results in prevention of bone loss or its normalisation if administration is delayed until animals are grossly symptomatic with respect to body weight loss. Administration of OPG does not appear to affect any of the parameters relating to the primary disease, such as body weight loss or inflammation of the large intestine. The effects of OPG appear to be targeted solely on bone.
Many investigators have established the prevalence of osteoporosis in the Crohn’s disease population, with occurrence in the ulcerative colitis population being less well established. This may be because Crohn’s disease is considered to be a more systemic disease than ulcerative colitis, involving the entire gastrointestinal tract with a longer premonitory course and more extraintestinal manifestations. We chose this mouse model of IBD to be representative of the Crohn’s disease variant of IBD and we did find significant and reproducible BMD loss. This occurred primarily in trabecular rather than cortical bone. Several theories have been proposed to explain the mechanism of action of BMD loss in Crohn’s disease. As none of the mice in this study received any corticosteroid or other immunosuppressive therapy, BMD loss is most likely a result of the primary disease, which is concordant with most of the published studies on Crohn’s disease associated osteoporosis in humans.

The use of an animal model allowed us to address the relative contributions of inflammation and/or malabsorption to BMD loss. Most studies in humans have concluded that nutritional factors, vitamin deficiency, and malabsorption are not major contributing factors to BMD loss. We found in this study that all mice consumed, on average, the same amount of food or water over the course of the study, and hence decreased food intake was not a causative agent. The question of whether or not in vivo malabsorption subsequently occurs in mice is more difficult. Several studies in animals have demonstrated that diets deficient in calcium and phosphorus or protein take months to cause BMD loss, and our study lasted only five weeks. The decrease in circulating PTH and the presence of a TNF-α elaborating cellular infiltrate in the bone suggests that BMD loss is due primarily to inflammatory processes. It is possible that the inflammation led to degradation and resorption of calcified bone matrix, causing an increase in serum calcium. This increase would cause a rapid decrease in circulating PTH levels which would serve to prevent further increases in serum calcium. This homeostatic mechanism appeared to be effective as serum calcium was unchanged in all of the mice in the study, regardless of disease or treatment state. OPG treatment almost normalised decreased PTH levels, possibly as a consequence of decreased bone destruction and release of less free calcium from bone. The presence of a TNF-α elaborating cellular inflammatory infiltrate in the bones of diseased animals is also consistent with this theory, as several investigators have shown a direct link between levels of secreted TNF-α and RANKL induced osteoclast mediated bone destruction. It has further been shown that RANKL can act to maintain inflammation. Although the reported data in human studies is inconsistent on this point, this theory of increased bone resorption due to proinflammatory cytokines, accompanied by decreased bone formation, is in broad agreement with the majority of the published studies. Consistent with this, bone histomorphometry in our study revealed that the diseased animals had more osteoclasts and significantly less osteoblasts compared with normal animals. The observed increases in serum TRAP (marker for osteoclasts) and decrease in serum ALP (marker for osteoblasts) are in agreement with this observation, although future studies to investigate the relative roles of inflammation and/or malabsorption are warranted.

Various therapeutic interventions, ranging from calcium supplementation or glucagon-like peptide 2 to the use of bisphosphonates have been tried to treat the osteoporosis associated with the collective IBD patient population but efficacy has ranged from absent to modest, respectively. There is still an acute need for effective targeted therapy for this extraintestinal manifestation of the disease. The use of OPG in this study provided significant protection against IBD associated BMD loss but did not have any significant effect on body weight loss, levels of SAA, or the disease associated haematological abnormalities or inflammation of the large intestine or the bone itself. This targeted effect of OPG is consistent with the many other preclinical studies performed. When OPG administration was delayed until the diseased animals had lost 10% of their body weight, OPG still normalised BMD of the tibiae. It is important to note that this reversal of osteoporosis was probably due to the fact that the animals were young and still undergoing longitudinal bone growth. In these animals, osteoclast inhibition would cause an exaggerated increase in BMD that would not likely be observed in skeletally mature humans. In this study we used ongoing prophylactic and therapeutic administration of OPG at a relatively high dose of 5.0 mg/kg three times per week. Other studies in animal models of autoimmune disease have shown that lower and less frequent doses may be equally effective.

On a cellular level, we found that OPG completely eliminated any detectable osteoclasts from the bone, even with a therapeutic dosing regimen, as well as eliminating any detectable osteoblasts. Elimination of osteoclasts was expected and elimination of osteoblasts is probably due to the tightly coupled regulation of these two cell types. Normalisation of serum TRAP by treatment with Fc-OPG reflects its linkage to levels of osteoclasts and the decrease in serum ALP by treatment with Fc-OPG is consistent with elimination of osteoblasts in bone.

In summary, this study suggests that inflammation is the primary cause of bone loss in IBD and that recombinant human osteoprotegerin may be of significant benefit in the clinical management of IBD associated osteopenia and osteoporosis.

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Toll-like receptor 4 gene in IBD: further evidence for genetic heterogeneity in Europe

There is now strong evidence implicating the enteric flora in the aetio-pathogenesis of inflammatory bowel disease (IBD), and identification of CARD15 (NOD2) as a pattern recognition receptor (PRR) has given novel insights into host-bacteria interactions. CARD15 is implicated as the intracellular sensor of muramyl dipeptide, a highly conserved bacterial peptidoglycan motif, and raises the question of whether other PRRs are involved in the pathogenesis of Crohn’s disease (CD).

Toll-like receptor 4 (TLR4), in combination with CD14, LBP, and MD-2, acts as the PRR for the lipid A moiety of lipopolysaccharide, a major component of gram negative bacteria. Two common cosegregating polymorphisms of this gene have been described in humans, Asp299Gly and Thr399Ile. Asp299Gly has been associated with reduced bronchial responsiveness following lipopolysaccharide stimulation although recent data have questioned the functional effect of this variant.1

We therefore note with interest the article from Franchimont and colleagues reporting the Asp299Gly frequency in a Belgian population with IBD (Gut 2004;53:987–92). Variant alleles were associated with both CD and ulcerative colitis (UC) in two cohorts and the allele was preferentially transmitted from carriers to affected subjects in a transmission disequilibrium test.

However, apparently contradictory data from elsewhere in Europe highlight the difficulties of interpretation of genetic association studies from single populations. Tokor et al examined the presence of both the Asp299Gly and Thr399Ile polymorphisms in a smaller German IBD population.2 In contrast with the Belgian data, this group identified an association of Thr399Ile with UC but there was no association with Asp299Gly, and no association with CD. In addition, we have previously published data on Asp299Gly in 480 Scottish patients with IBD and found no association with either CD or UC.3

Why are these data sets discrepant? Issues relating to statistical power, population stratification in case control studies, and phenotypic heterogeneity within IBD may contribute but we suggest more detailed examination of these data (table 1) provides evidence for genetic heterogeneity between populations in Europe.

CD genotype frequencies were very similar in the Leuven and Edinburgh data sets, and not significantly different from the German CD results. However, allelic frequencies in healthy controls were significantly different in the Scottish population compared with European controls (8.8% v 4.6%; p = 0.008, odds ratio 1.47 (confidence interval 1.2–3.5)).

It is clearly relevant that a strong suggestion of heterogeneity between populations was given in the original description of Arbour and Lorenz’ where control population allelic frequencies for Asp299Gly ranged from 3.3% to 7.9% in French and North American data sets. It is noteworthy that these allelic variants were absent in the Japanese population.4

Moreover, there is now compelling evidence for genetic heterogeneity between populations for CARD15 in both healthy controls and CD patients. Variant alleles are absent from Asian CD and control populations and exist at a lower frequency in African American CD patients and Ghanaian controls (carriage 1%).5 Carriage frequencies in CD patients approaching 50% have been documented in Caucasian populations from North American and Central Europe. There is also striking evidence for heterogeneity within Europe, and evidence for a geographical North-South gradient in gene effect, as for the CFTR delta 908 mutation in cystic fibrosis. Lower CARD15 (NOD2) frequencies in CD have been reported from Scotland6 and Finland and are absent in a small Icelandic population.7

These data illustrate further the real difficulties in candidate gene analysis in complex diseases. It is likely that the contribution of individual genetic determinants will differ between populations. We suggest that further genetic (as well as functional) data are required before the exact contribution of inherited variants of the TLR4 gene can be confirmed.

References

Author’s reply

We would like to thank Arnott et al for their interesting comments. Needless to say, we all agree that great caution should apply when interpreting positive or negative association studies because of issues such as sample size, cryptic population substructure, and phenotype misclassification. To this end, a transmission disequilibrium test (TDT) should always be performed to alleviate skepticism and doubts. It is true that a clear genetic heterogeneity emerges when examining NOD2 and toll-like receptor 4 (TLR4) carriage frequencies in patients with Crohn’s disease in Europe, North America, and Asia. That Crohn’s disease is a heterogeneous and polygenic disease is obvious in the light of recent genome wide screens. However, if multiple genes contribute to Crohn’s disease, their relative impact may vary from one phenotype to another (or from one Crohn’s

Table 1 Number of patients studied and Toll-like receptor 4 Asp299Gly allele and genotype frequencies in Crohn’s disease (CD), ulcerative colitis (UC), and healthy control (HC) populations from Leuven, Munich, and Edinburgh

<table>
<thead>
<tr>
<th>Patient numbers (allele frequencies)</th>
<th>CD</th>
<th>UC</th>
<th>HC</th>
<th>Crohn’s disease genotype frequencies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WC</td>
<td>HZ</td>
<td>HZ</td>
<td>Wild-type</td>
</tr>
<tr>
<td>Leuven</td>
<td>334 (11.0%)</td>
<td>163 (10.0%)</td>
<td>139 (5.0%)</td>
<td>79.3</td>
</tr>
<tr>
<td>Munich</td>
<td>102 (7.0%)</td>
<td>98 (9.0%)</td>
<td>145 (4.0%)</td>
<td>85.0</td>
</tr>
<tr>
<td>Edinburgh</td>
<td>234 (10.3%)</td>
<td>246 (6.8%)</td>
<td>189 (8.8%)</td>
<td>79.5</td>
</tr>
</tbody>
</table>

HC allele frequencies differed between Edinburgh and Munich (p=0.02, odds ratio 1.35 (confidence interval 1.1–4.5)) and those between Edinburgh and Leuven approached significance (p=0.06).
delivered to the pulmonary circulation are being metabolised locally in these patients. Indeed, in experimental cirrhosis, there is evidence of increased production of endothelin type B receptors in the pulmonary circulation, and these are responsible for clearance of ET-1. Therefore, although our single measurement of ET-1 in the pulmonary artery does not reflect the net result of ET-1 metabolism in the pulmonary vasculature, it does represent the amount of ET-1 that is delivered to the pulmonary artery, potentially causing pulmonary vasoconstriction.

With respect to measurement of ET-1 across the splanchnic circulation, there is already ample evidence in the literature to support increased production of ET-1 in the splanchnic circulation as well as in the liver. Therefore, it is reasonable to postulate that the increased ET-1 levels in the pulmonary artery, which is downstream from the splanchnic circulation, is largely derived from the increased production in that circulation.

Pellicelli’s proposal of interleukin 6 (IL-6) being one of the mediators involved in the pathogenesis of portopulmonary hypertension in cirrhosis is an interesting one. IL-6 can increase platelet production in small muscular arteries and capillaries as well as enhancing platelet activation. In addition, IL-6 promotes the coagulation cascade without affecting fibrinolysis, causing fibrin thrombi. If this occurs in the pulmonary circulation, then pulmonary hypertension can develop. Similar to what has been reported in the literature,7 Pellicelli and his group have found increased levels of IL-6 in patients with cirrhosis. However, it is not clear whether the increased plasma levels of IL-6 in cirrhosis are a non-specific inflammatory response in patients who are generally ill or part of a pathogenetic mechanism of some complication of cirrhosis. To date, there is no firm evidence that IL-6 is involved in the pathogenesis of either primary or secondary pulmonary hypertension. The challenge remains for us to find increased IL-6 levels in the pulmonary circulation in patients with portopulmonary hypertension before it can be implicated in the pathogenesis of this serious but uncommon complication of cirrhosis.

We present a complication seen in two of our patients, with no consequences to their health or management, but with an impact on examination accuracy and usefulness: oesophageal entrapment in an extra oesophageal vascular compression.

Patient 1 was a 74 year old man with a history of a prosthetic metallic mitral valve and congestive heart failure who had suffered three episodes of obscure overt gastrointestinal bleeding. In his community hospital he had undergone upper and lower endoscopy twice, radioisotope bleeding scans, magnetic resonance imaging angiography, and conventional angiography but the source of the gastrointestinal bleeding was not found. He needed oral iron supplementation but this did not correct his iron deficiency anaemia. Previous radiographic contrast studies of the small bowel had been normal. It was then referred to our unit in the distal endoscopy examination. After the patient swallowed the capsule, we could see that without an apparent intrinsic stricture, the capsule was retained in the second third of the oesophagus for approximately four hours, progressing to the stomach after this time with no apparent manoeuvre or fluid ingestion (fig 1). The study could not be completed to the terminal ileum because the batteries became exhausted at the level of the proximal ileum. Nevertheless, we could see three bleeding jejunal ulcers as the cause of his gastrointestinal bleeding.

Our second patient was a 72 year old woman with a mitral prosthetic valve and chronic auricular fibrillation. She had undergone upper and lower gastrointestinal endoscopy and mesenteric angiography because she had passed dark stools with severe anaemia, on repeated occasions. A plain chest x ray showed marked cardiomegaly. She was referred to our centre for a small bowel capsule endoscopy examination. The capsule got stuck at a pulsate area in the distal oesophagus, staying there for up to three hours and passing afterwards without a specifically related cause (fig 2). The study
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References

Endogenous heparinoids in acute variceal bleeding
The risk of variceal bleeding in cirrhosis is associated with increasing liver dysfunction, larger varices, endoscopic red signs, and higher portal pressure. However, why bleeding occurs unpredictably and infrequently in individual patients is unknown.

Bacterial infections occur in 35–66% of cirrhotics presenting with gastrointestinal bleeding.\(^1\) We proposed a possible pathophysiological basis linking infection and variceal bleeding via endotoxin induced endothelin release and subsequent portal pressure rise, combined with impaired platelet aggregation due to endotoxin induced nitric oxide and prostacyclin.\(^2\) Infected cirrhotics demonstrate a heparin effect using heparinase I modified thromboelastography (TEG) and have anti-Xa activity.\(^3\) Now we show similar findings in two cirrhotics during the course of acute variceal bleeding.

Patient 1 was male, 66 years old (Child-Pugh grade C), and patient 2 was female, 42 years old (Child-Pugh grade B), both with alcoholic cirrhosis. Both received endoscopic banding, intravenous terlipressin, and cefotaxime prophylactically as currently recommended.\(^4\) Baseline bacterial screens were negative with no subsequent infections. Blood samples after informed consent were taken at baseline (before any therapy) and subsequently over seven days. Heparinase I modified and standard TEG (Haemoscope Corp., Skokie, Illinois, USA) were performed simultaneously using only calcium activated citrated blood from the sample 90 minutes after venepuncture: a heparin effect was defined as an improvement in \(r\) time, \(k\) time, and \(\alpha\) angle occurring together. Anti-Xa was assessed by chromogenic (Sigma Diagnostics, Poole, Dorset, UK) and clotting assays (Diagnostic Reagents, Thame, Oxford, UK).

A heparin effect was detected between one hour (patient 2) and six hours (patient 1) after the initial bleeding episode and persisted for 6–7 days, not fully corrected by fresh frozen plasma and/or red blood cells (fig 1) given during the first 24 hours. In patient 1, anti-Xa activity was positive during the same time span in which there was a heparin effect.

Evaluated TEG parameters were “\(r\) time” (time for the clot to start forming), “\(k\) time” (time between the TEG trace reaching 2 mm and 20 mm), and “\(\alpha\) angle” (the slope drawn...
from the r to the k value). These worsened over time: in patient 1, r time 11 minutes (four hours after the bleeding episode) to 18.8 minutes (six hours later); k time from 2.7 minutes to 7.3 minutes; and α angle from 53.8° to 28.7°. In patient 2, r time increased from 11.2 minutes (one hour after the first haematemesis) to 33.8 minutes (two hours after the beginning of the haemorrhage) and disappeared over five days, over the same time course of antibiotic therapy. This was documented shortly after the beginning of the haemorrhage and disappeared over five days, over the same time course of antibiotic therapy. This was also seen by Montalto and colleagues. The absence of a demonstrable heparin effect at the beginning of bleeding and appearance thereafter could suggest that bleeding is a cause of its occurrence, and not the other way round. However, citrated blood may mask an initial less severe heparin effect and this needs to be evaluated compared with native blood. The heparin effect could influence continued varicel bleeding or early rebleeding. It is possible that the heparin effect might be independent of the absence of antibiotics. This phenomenon deserves wider study, particularly as bacterial infection has been linked to failure to control varicel bleeding and early rebleeding, in a randomised study of prophylactic antibiotics.4

References

Changing epidemiology of IPSID in Southern Iran

Some 40 years ago physicians in the Middle East noted a high incidence and prevalence of upper small intestinal disease. Later this condition was found to be associated with malabsorption as well as the presence of alpha heavy chain proteins. The disease was named by the WHO as “immunoproliferative small intestinal disease” (IPSID). One of the earliest reports of IPSID was from our centre. This study was designed to confirm the trend in the epidemiology of IPSID over the past 25 years in our medical centre. In a retrospective study (March 1974 to March 1999), we reviewed pathology reports from all surgical pathology laboratories in the province of Fars located in Southern Iran.

All reports, which were labelled as IPSID, were reviewed by the authors. Cases were grouped into five year intervals according to the date of the initial diagnosis and five year age groups. Age specific rates were calculated using midperiod population denominators for each age group, and summary age adjusted incidence rates were calculated by direct standardisation using the world standard population.

During this 25 year period, more than 500 000 surgical pathology reports were recorded. There were 5421 gastrointestinal tract cancers of which 2326 (43%) were gastric cancers, 1398 (26%) colon cancers 1161 (21%) oesophageal cancers, and 536 (10%) small bowel cancers. Of the small bowel cancers, 161 (30%) cases were IPSID. This composed 3% of all gastrointestinal cancers in this period.

Among the 161 IPSID cases, 98 (61%) were males with a mean age of 31.74 (SD 14.94) years and 63 (39%) were females (mean age 26.85 (8.88)). The standardised rate ratio (95% confidence interval) of males to females in the study was 1.39 (1.26, 1.69), which represents a higher incidence of IPSID in males. Almost all cases were village dwellers or those who had recently immigrated to large cities from their villages. Age specific rates and absolute frequency of IPSID in males and females are shown in table 1. This disease had its highest incidence in the third decade of life in both sexes. There has been a persistent decrease in the incidence of IPSID since 1986, as shown in fig 1.

The sharp decrease in the incidence of IPSID in the period 1978-1983 coincided with the time of revolution and the Iraq-Iran war, which caused instability in all organisations. The incidence of IPSID has decreased over the past 15 years (r² = 0.26, t (14) = –2.25, p = 0.04).

IPSID was once the most common small intestinal malignancy in the Middle East. Early infectious stress in infancy and chronic antigenic stimulation in the earlier part of life along with genetic factors are probably important in the pathogenesis of IPSID. In our series of 161 patients with IPSID, we observed a dramatic decrease in the incidence of the disease over the past decade. After the Islamic revolution in Iran, improving sanitation in villages was one of the priorities of the many health strategies in Iran. Access to sanitary drinking water in rural areas increased from 35% before 1988 to 90% a decade later. Vaccination programmes increased dramatically after the Islamic revolution, reaching more than 90% of children. Local health facilities increased dramatically during the first two decades after the revolution.

Table 1 Age specific rates (ASR) and absolute frequency of immunoproliferative small intestinal disease in males and females in different age groups in the Fars province, Iran, from 1974 to 1999

<table>
<thead>
<tr>
<th>Age group (y)</th>
<th>Total ASR</th>
<th>Males ASR</th>
<th>Females ASR</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-4</td>
<td>2</td>
<td>0.02</td>
<td>1</td>
</tr>
<tr>
<td>5-9</td>
<td>6</td>
<td>0.05</td>
<td>6</td>
</tr>
<tr>
<td>10-14</td>
<td>5</td>
<td>0.05</td>
<td>4</td>
</tr>
<tr>
<td>15-19</td>
<td>15</td>
<td>0.17</td>
<td>4</td>
</tr>
<tr>
<td>20-24</td>
<td>35</td>
<td>0.54</td>
<td>14</td>
</tr>
<tr>
<td>25-29</td>
<td>35</td>
<td>0.59</td>
<td>17</td>
</tr>
<tr>
<td>30-34</td>
<td>11</td>
<td>0.25</td>
<td>7</td>
</tr>
<tr>
<td>35-39</td>
<td>16</td>
<td>0.43</td>
<td>11</td>
</tr>
<tr>
<td>40-44</td>
<td>10</td>
<td>0.38</td>
<td>11</td>
</tr>
<tr>
<td>45-49</td>
<td>10</td>
<td>0.42</td>
<td>7</td>
</tr>
<tr>
<td>50-54</td>
<td>7</td>
<td>0.32</td>
<td>7</td>
</tr>
<tr>
<td>55-59</td>
<td>5</td>
<td>0.28</td>
<td>3</td>
</tr>
<tr>
<td>60-64</td>
<td>2</td>
<td>0.12</td>
<td>2</td>
</tr>
<tr>
<td>&gt;65</td>
<td>2</td>
<td>0.13</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>161</td>
<td>0.21</td>
<td>98</td>
</tr>
</tbody>
</table>

Age specific rate per 100 000 population in each age group.

Figure 1 Incidence rate of immunoproliferative small intestinal disease (IPSID) in the Fars province, Iran, from 1983 to 1999.
We postulate that improvement in health in general and decreasing childhood gastroenteritides in particular has resulted in a decrease in the incidence of IPSID. This report highlights the almost complete disappearance of a malignant disease from a region where it was once very common. This is probably related to changes in environmental factors, decreasing exposure to infectious agents.

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References

Microscopic (collagenous and lymphocytic) colitis triggered by food allergy

Collagenous and lymphocytic colitis are rare diseases of unknown aetiology but several issues, in particular the good response to budesonide, are suggestive of immunopathology. Patients have watery diarrhoea without abnormal findings on colonoscopy but with increased numbers of intraepithelial lymphocytes, mast cells, and eosinophils on histological examination.

We report six patients seen between 1993 and 1999 who were first diagnosed as having collagenous/lymphocytic colitis. Signs of allergy entailed a work up for food allergy. (table 1).

All patients were investigated with skin prick testing, total and allergen specific IgE with food, and environmental allergens. Excretion of urine methylhistamine (UMH) was measured on a normal and hypoallergenic potato–rice diet. Colonoscopy with endoscopically guided segmental lavage for intestinal IgE was carried out and biopsies were investigated by routine pathology (haematoxylin–eosin), immunohistochemistry for eosinophil peroxidase, and amount of eosinophil cationic protein and tryptase in the whole biopsy. Clinical activity was mainly assessed by number of stools/day and the Karnofsky index for general performance.

After allergen identification, all patients were counselled on elimination of the allergen, except for one case where no allergen was identified. In this case, in a second patient with multiple sensitisations, and in a third with allergy to basal foods, additional cromolyn therapy was initiated. A trial of hypoallergenic diet and subsequent controlled addition of food with low allergenicity was performed. Additional antihistaminergic therapy (fexofenadine) was recommended as supplementary therapy for periods of exacerbation.

As all patients were followed prospectively every 12 months after diagnosis (outpatient clinic and structured telephone interview) and all had symptom reduction in terms of stool frequency and consistency (table 2). General performance was completely restored in four patients and improved in one. The remaining patient was still very restricted in his activities due to incapacitating coronary artery disease but his gastrointestinal symptoms are tolerable.

Another patient was not willing to undergo colonoscopy for lavage and allergen identification, nor willing to quit smoking. His stools normalised after a six month course of cromolyn but he still suffers from bouts of diarrhoea during stress. He considers his general performance as good.

Histology was available for one patient before and after therapy. After dietary elimination, eosinophil infiltrate was markedly less dense and degranulated.

The mechanisms of diarrhoea in collagenous colitis include a pronounced diffusion barrier with diminished net absorption of sodium and chloride ions. Allergens could induce increased eosinophil infiltration and enhance transforming growth factor β with increased collagen deposition. Eosinophils are highly susceptible to steroids which may explain the good response of collagenous colitis to budesonide.

In summary, a subgroup of patients with microscopic colitis suffer from food allergy. Further work up for allergy is sensible in those patients with a history of atopic disease or blood/tissue eosinophilia. Allergen elimination can decrease or abolish the need for medication. Antiallergic therapy can be added to the therapeutic regimen.

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Table 2 Number of stools per day (Baerts score) before and after therapy

<table>
<thead>
<tr>
<th>Sex</th>
<th>Age (y)</th>
<th>Follow up (y)</th>
<th>Atopy</th>
<th>Signs of allergy</th>
<th>Diagnostic markers suggestive of allergy</th>
<th>Identified allergen</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>49</td>
<td>5</td>
<td>+</td>
<td>Improvement by hypoallergenic diet</td>
<td>Eos in bx</td>
<td>Egg, pollen, house dust mite</td>
</tr>
<tr>
<td>M</td>
<td>45</td>
<td>5</td>
<td>–</td>
<td>Urticaria, improvement by cromolyn</td>
<td>UHM</td>
<td>Spices, moulds, flours, celery, nuts, milk, maize, rice, apple, celery, soy</td>
</tr>
<tr>
<td>M</td>
<td>46</td>
<td>5</td>
<td>–</td>
<td>Painsinusitis</td>
<td>Eos in bx</td>
<td>Maize starch</td>
</tr>
<tr>
<td>M</td>
<td>52</td>
<td>3</td>
<td>–</td>
<td>IgG deficiency</td>
<td>Eos in bx</td>
<td>Milk, egg, soy</td>
</tr>
<tr>
<td>M</td>
<td>71</td>
<td>9</td>
<td>+</td>
<td>Prick</td>
<td>Prick, RAST</td>
<td>Ananas</td>
</tr>
<tr>
<td>F</td>
<td>59</td>
<td>4</td>
<td>+</td>
<td>Prick</td>
<td>UHM</td>
<td></td>
</tr>
</tbody>
</table>

Eos in bx, eosinophils in colon biopsy; UHM, urine methylhistamine.
Acetic acid spray in colonoscopy: an alternative to chromoendoscopy

We read with interest the article by Rutter et al (Gut 2004;53:256–60) and the letter in response to this article by Hata et al (Gut 2004;53:1722). Rutter demonstrated the advantage of magnifying chromoendoscopy using indigo carmine for detection of dysplasia compared with conventional colonoscopy without dye spray by back to back chromoendoscopy in patients with longstanding ulcerative colitis.

Hata et al discussed the characteristics and correct selection of dyes. In particular, Hata et al emphasised that there are two types of dye spraying: the contrast method in which dye is used solely to contrast the irregularity of the surface, and the staining method in which dyes such as crystal violet and methylene blue are used to stain the colonic mucosa. The latter technique provides more detailed structure of neoplastic as well as non-neoplastic colonic mucosa, which may contribute to more precise diagnosis of dysplasia and inflammatory change than the contrast method.

However, as Hata et al pointed out, a disadvantage of the staining method is that it is time consuming. Usually, it takes two or three minutes to stain one region. Therefore, it would be beneficial if there were an agent that demonstrated the fine structure of the colonic mucosa without a delay.

Recently, we have introduced acetic acid spray in screening colonoscopy to visualise the fine structure of colonic neoplasia, in which approximately 5 ml of 2% acetic acid solution is sprayed towards the targeted lesion in the same manner as for indigo carmine. The advantages of using acetic acid spray as a staining method are as follows.

Firstly, the fine structure of the mucosa can be demonstrated immediately (fig 1A, 1B).

Therefore, it reduces the time for examination, especially in patients with multiple lesions. Secondly, acetic acid effectively removes surface mucous material that interferes with magnifying observations (fig 1C, 1D). Lastly, acetic acid is less expensive.

We agree with Hata et al that it is essential to understand the various methods of dye spray and to apply them appropriately, according to the situation. Here, we advocate acetic acid spray as an alternative to dye spray for enhancing the fine structure of the mucosa. Hata et al titled their letter “To dye or not to dye. That is beyond question!” We would like to add “To spray dye or to spray acetic acid. That is our question!

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Conflict of interest: None declared.

References


Infliximab failure in cap polyposis

Cap polyposis is a rare condition that predominantly affects the rectosigmoid with distinctive clinicopathological features. The common symptoms are mucoid and bloody diarrhoea with abdominal pain and tenesmus. At endoscopy, polyps are red, sessile, and located at the apices of enlarged transverse mucosal folds with a normal intervening mucosa. Microscopic features include elongated hyperplastic looking glands with a mixed inflammatory infiltrate in the lamina propria. A cap of fibrinopurulent exudate covers the polyps. Treatment of this condition remains empiric. Metronidazole and steroids have been effective in some cases. Symptoms are often relieved by polypectomy but rectosigmoid resection may be required to control diarrhoea.

Some years ago, we reported in Gut on a 52 year old woman who needed sigmoid resection for cap polyposis. Following surgery, she did well until 1998 when she again complained of abundant mucoid diarrhoea with severe postprandial abdominal pain requiring daily antispasmodic therapy. Endoscopy with histology displayed the characteristic features of recurrent cap polyposis in the rectum (fig 1). This was again refractory to multiple therapies, including several courses of mesalamine, antibiotics, steroids, and laser photococagulation of polyps. At that time, we were aware of a case of cap polyposis that was successfully treated with infliximab. This was a 36 year old woman who had a one year history of cap polyposis and who experienced complete clinical, endoscopic, and histological remission following four infliximab infusions at eight week intervals. This encouraging observation led us to treat our patient with infliximab. Two infusions of infliximab 5 mg/kg were administered at four week intervals. In order to gain some insight regarding the potential involvement of tumour necrosis factor α (TNF-α) in this condition, TNF-α mRNA was measured in the
rectal mucosa using real time polymerase chain reaction before and after treatment, and compared with control values. Unfortunately, no clinical or endoscopic improvement occurred following infliximab infusions. TNF-α levels in the mucosa were not different compared with controls before and after treatment.

The reason for the discrepancy between this failure and the spectacular improvement observed by Bookman and colleagues is unclear. Our patient suffered from this condition for more than 10 years and may have had a more refractory form of the disease. One should also remember that in the case described by Bookman and colleagues, no control was available and spontaneous regression of cap polyposis has already been observed. The pathogenesis of cap polyposis remains unclear. Our data do not support the hypothesis that TNF-α plays a role in the pathogenesis of cap polyposis. An infectious or ischaemic aetiology has been suspected. Histological features similar to cap polyposis have been described in other disorders where mucosal prolapse is the underlying mechanism such as solitary rectal ulcer syndrome or prolapsed colostomies. It has therefore been suggested that abnormal colonic motility may be an important aetiological factor.

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References

Differential modulation of p38 mitogen activated protein kinase and STAT3 signalling pathways by infliximab and etanercept in intestinal T cells from patients with Crohn’s disease

There is growing evidence that the efficacy of anti-tumour necrosis factor α (TNF-α) therapies in Crohn’s disease (CD) may critically depend on the binding of the transmembrane precursor of TNF-α (mTNF-α), thus eliciting complex intracellular signalling events, a process described as “reverse signalling.” It has also been suggested that failure of another TNF binding agent, etanercept (Enbrel; a recombinant TNFR2:Fc fusion protein), to induce peripheral and lamina propria lymphocyte apoptosis in CD patients via a caspase dependent mechanism, further corroborating the findings of previous studies. Thus it has been suggested that abnormal colonic motility may be an important aetiological factor.

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Differential modulation of p38 mitogen activated protein kinase and STAT3 signalling pathways by infliximab and etanercept in intestinal T cells from patients with Crohn’s disease

There is growing evidence that the efficacy of anti-tumour necrosis factor α (TNF-α) therapies in Crohn’s disease (CD) may critically depend on the binding of the transmembrane precursor of TNF-α (mTNF-α), thus eliciting complex intracellular signalling events, a process described as “reverse signalling.” It has also been suggested that failure of another TNF binding agent, etanercept (Enbrel; a recombinant TNFR2:Fc fusion protein), to induce peripheral and lamina propria lymphocyte apoptosis in CD patients via a caspase dependent mechanism, further corroborating the findings of previous studies. Thus it has been suggested that abnormal colonic motility may be an important aetiological factor.
However, the authors do not discuss other signalling pathways that are activated via ligation of transmembrane TNF-α by infliximab (for example, we have shown that infliximab also transiently activates p38 mitogen activated protein kinase (MAPK) in monocytes in vitro and in the lamina propria of CD patients in vivo).2,3 Responders and non-responders to infliximab differ in the pattern of mucosal p38MAPK target phosphorylation, but not caspase-3 activation, further emphasizing the complex modulation of intracellular signalling pathways beyond mere neutralisation of TNF-α.4 To show if these signalling pathways are also activated in primary T cells, we analysed the influence of infliximab and etanercept on p38MAPK activation and apoptosis in an established model of non-transformed in situ activated T lymphocytes.5,6 According to the findings of van den Brande et al.,1 we observed PARP cleavage as a molecular hallmark of apoptosis in cultures grown with infliximab (fig 1A) but not in the presence of etanercept. Whereas no increase in phosphorylated p38MAPK could be detected after etanercept stimulation, significant activation (that is, dual phosphorylation of p38) after 24 hours after infliximab treatment was observed in 4/5 of the cell lines derived from CD patients (fig 1A).

We have demonstrated previously that constitutive tyrosine phosphorylation of the transcription factor signal transducer and activator of transcription 3 (STAT3) may represent a specific feature of intestinal T cells from Crohn’s disease.7 Tyrosine phosphorylated STAT3 can be found in CD4+ cells in the inflamed mucosa, as shown by immunofluorescence analysis (fig 1B). Surprisingly, infliximab and etanercept were able to reduce STAT3 phosphorylation in T cells from CD patients (cultured with interleukin (IL)-2 and IL-4) to a similar extent (fig 1B). It is tempting to speculate that the previously reported downregulation of interferon γ/granulocyte macrophage-colony stimulating factor by both infliximab and etanercept is also involved in the decrease in STAT3 tyrosine phosphorylation as both cytokines are potent inducers of STAT3 activation via Janus kinases. Mechanistically, this common action of infliximab and etanercept is due to neutralisation of an autocrine loop of constitutively released sTNF-α. Although the findings suggest that inhibition of cytokine dependent inducible STAT3 phosphorylation could be dispensable for therapeutic efficacy in CD, aberrant constitutive STAT phosphorylation in T lymphocytes may still have an important modulatory role in chronic intestinal inflamma-

These observations corroborate the hypothesis that TNF-α binding agents may exert distinct functions on lymphocyte activation and survival, either by “reverse signalling” via binding of mTNF-α resulting in p38MAPK activation and apoptosis or by neutralisation of sTNF-α, which in this model may serve to inhibit STAT3 signalling. The individual properties of TNF-α blockers to induce either of these molecular actions may be differentially responsible for therapeutic success or failure in chronic inflammatory disorders such as rheumatoid arthritis, psoriasis, or CD. In CD, ligation of mTNF-α, subsequent apoptotic processes, and MAPK signalling seem to be critically required. Molecular dissection of mTNF-α apoptotic and non-apoptotic signalling may have important implications for the design of future therapeutic strategies in inflammatory bowel disease.

References

Author’s reply
We thank Rosenstiel et al for their interest in our paper regarding the role of the chimeric anti-tumour necrosis factor (TNF) α antibody, infliximab, in reverting defective lamina propria T cell apoptosis in Crohn’s disease via a caspase dependent pathway (Gut 2004;53:70–7).

In their interesting letter, Rosenstiel et al investigate the complex TNF-α blocker machinery in Crohn’s disease by analysing the influence of infliximab and recombinant TNF receptor/immunoglobulin G fusion protein, etanercept, on p38 mitogen activated protein kinase (MAPK) activation, apoptosis, and signal transducer and activator of transcription 3 (STAT3) tyrosine phosphorylation in lamina propria T cells from steroid resistant Crohn’s disease patients. Infliximab, but not etanercept, has been shown to result in p38MAPK activation and apoptosis, while both of these agents were able to reduce STAT3 phosphorylation in Crohn’s disease lamina propria T cells.

If data on the differences in mucosal T cell apoptosis inducing capacity of infliximab and etanercept are in keeping with the results obtained by van den Brande and colleagues, the observation that infliximab induces activation of a proinflammatory MAPK does not tally with the evidence that the guanylyl hydrasone c-Jun N terminal kinase/p38 inhibitor CNI-1493 induces clinical improvement in steroid resistant Crohn’s disease patients.3 The reason for this discrepancy probably lies in the complex and dichotomal role of p38MAPK in inflammatory signal transduction. p38MAPK inhibition is efficacious in chronic inflammatory disease but not in acute experimental colitis, where it causes adverse effects,7 and it shares differential actions in different T cell subpopulations.8 In naïve T cells, in fact, the p38 inhibitor SB203580 inhibits proinflammatory cytokine production whereas in T helper type (Th) 1 cells it does not affect TNF-α release but strongly impairs anti-inflammatory interleukin 4 production, thus shifting the balance between Th1 and Th2 cytokines to the former.9

Since several lines of evidence have also suggested the proapoptotic role of the p38 pathway,10 Waetzig and colleagues have postulated that the increased phosphorylation of the p38MAPK downstream effector ATF-2, found after infliximab treatment only in responder Crohn’s disease patients, could enhance infliximab induced apoptosis of immune cells in this subgroup of patients. However, the similar proportion of lamina propria immune cell apoptosis after infliximab treatment in both responders and non-responders suggests that differential signal transduction downstream of p38MAPK are not related to infliximab induced immune cell apoptosis.11

Disturbances in STAT signalling pathways, which can transduce the inflammatory signals, messages of most of cytokines/cytokine receptors, have been shown to be involved in the pathogenesis of Crohn’s disease. In particular, STAT1 and STAT3 proteins are constitutively activated in lamina propria CD4+ T cells from active Crohn’s disease patients.12 The finding of Rosenstiel et al that both infliximab and etanercept are able to reduce in vitro STAT3 tyrosine phosphorylation in Crohn’s disease lamina propria T cells is probably related to the capacity of both of these agents to neutralise soluble TNF-α. Interestingly, the absence of quantitative differences in the p38MAPK blocker etanercept and etanercept in downregulating STAT3 signalling is consistent with the data of van den Brande and colleagues who have shown that both drugs can neutralise soluble TNF-α to a similar extent.

Taken together, these findings suggest that both the apoptotic and non-apoptotic intra-cellular signalling pathways underlying the therapeutic benefit of anti-TNF-α strategies in Crohn’s disease are multifaceted and more complex than initially thought. Dissecting the MAPK signalling cascades that are selectively activated in the abnormal immune response
BOOK REVIEW

Small and Large Intestine

The Small and Large Intestine, one of four volumes in this series named the Requisites in Gastroenterology edited by Anil Rustgi of the University of Pennsylvania, provides a refreshing readable overview. While successfully avoiding entrenched in detailed consideration of the scientific literature, it provides a mainstream viewpoint of the major issues. It is presented in a logical and clear manner aimed at achieving an understanding at a basic level which will provide the reader with a good platform to focus on specific areas with more in depth study. In this series, Rustgi moves away from an account of the substantial gastroenterological literature which often includes clinical trials with apparently contradictory conclusions that may lead to controversies unwanted by the novice. Rather than being expected to weigh up the evidence, the student is more usually interested in the general viewpoint of the main body of experts. This series avoids the comprehensive consideration of the literature often found in the larger reference texts and concentrates on the delivery of practical guidance and the acquisition of a general grasp of the subject, particularly for physicians in training and medical students.

Each of the 13 chapters have well defined formats, orientating the reader with a brief initial chapter outline and general introduction to the subject. The majority then discuss the epidemiology and pathophysiology before providing a more detailed account of clinical evaluation and treatment. Considerable attention is given to pragmatic clinical management issues cutting through much of the academic detail to provide practical information, useful to those considering the day to day issues of gastroenterology. For example, in the Crohn’s disease chapter, there are two pages dedicated to pathogenesis, five to clinical assessment and diagnosis, followed by 16 pages on therapy of which only one relates to surgery, and that includes a table (clearly we are heading in the right direction with therapy!). However, nutritional therapy is given very little mention, which may reveal subtle differences in the character of medical gastroenterology between the USA and Europe.

Most chapters stay well within general dogma, including the most important, latest, and robust advances in knowledge; a few have a particular emphasis. The “irritable bowel syndrome” chapter develops the psychosocial/psychiatric approach to patients to a degree that might reflect the chapter author’s own clinical experience as a proponent of psychiatry and medicine. It nevertheless addresses the other pathogenic aspects of irritable bowel syndrome, although promotes rather strict Rome criteria to the diagnosis which perhaps does not describe the full range of irritable bowel syndrome patients seen in the average outpatient clinic and is of more value if a well defined group of patients is required, such as for conducting clinical trials. A positive diagnosis based on irritable bowel syndrome symptoms is also recommended. This should be tempered with caution as there are many pitfalls associated with this particular group of patients, particularly for those less experienced in this field of practice who are the target audience for this textbook. The chapters on “intestinal polyposis syndromes and hereditary colorectal cancer” and “colorectal neoplasia”, perhaps by necessity, stray somewhat from the clinical emphasis to include a more detailed account of the pathogenesis and epidemiology. They cover the basic genetic aspects in rather more detail than is the trend in other chapters and offer more discussion of the evidence base rather than being confined to expert interpretation and opinion. This perhaps deflects from the needs of the intended target audience towards those more familiar with the subject. Unlike the other chapters, there is also a degree of overlap between these two chapters, which is particularly related to the clinical and genetic criteria for diagnosis of HNPCC and the polyposis syndromes, and the criteria for genetic testing and screening. Both chapters are well written and interesting but it might have been more in keeping with the aims of the book to have combined them and kept to a more clinical approach. The weight of the text is augmented by key points in boxes which summarise areas of particular importance covered in each chapter, thus providing a useful at a glance reference. Several chapters contain investigation and treatment algorithms. These are particularly useful in the chapters on diarrhoea, inflammatory bowel disease, and colorectal neoplasia, and would have been a welcomed addition to other areas, particularly the investigation of malabsorption.

There is a strong sense that this text has been prepared with certification and recertification of North American Physicians in mind but it is also well suited to medical students in the broader sense, perhaps revising for finals, MRCP candidates, and medical registrars in training. Although not referenced, each chapter offers a guide to further reading providing a useful introduction to the scientific literature. In this volume, editors Lichtenstein and Wu achieve the aims of the series, as indicated by Anil Rustgi, the editor in chief in his foreword, to provide a user friendly text, imparted with expert knowledge and insights, that together constitute an overview and refresher course aimed at those training in the field of gastroenterology and those in other areas of medicine who want a succinct pragmatic overview.

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CORRECTION

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The spelling of one of the authors in the paper by Byrne et al (CD4+CD45RB+B T cell transfer induced colitis in mice is accompanied by osteopenia which is treatable with recombinant human osteoprotegerin, 2005;54:78–86), published in the January 2005 issue, was incorrect. R Manoukian should read R Manoukian.