INFLAMMATORY BOWEL DISEASE

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

F R Byrne, S Morony, K Warmington, Z Geng, H L Brown, S A Flores, M Fiorino, S L Yin, D Hill, V Porkess, D Duryea, J K Pretorius, S Adamu, R Manuokian, D M Danilenko, I Sarosi, D L Lacey, P J Kostenuik, G Senaldi

Gut 2005;54:78–86. doi: 10.1136/gut.2003.035113

Background and aims: Transfer of CD4⁺CD45RB^{Hi} 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^{Hi} or CD4⁺CD45RB^{Lo} T cells (4×10^5) 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^{Hi} T cells developed osteopenia (as indicated by decreased bone density accompanied by decreased osteoblasts and increased osteoclasts) and colitis (as indicated by histological changes in the large intestine). Mice that received CD4⁺CD45RB^{Lo} 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 with Fc-OPG increased bone density in mice that received either CD4⁺CD45RB^{Hi} or CD4⁺CD45RB^{Lo} 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^{Hi} T cell recipients was accompanied by hypoparathyroidism that was partially normalised by treatment with Fc-OPG. CD4⁺CD45RB^{Hi} 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^{Hi} 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.

See end of article for authors' affiliations

Correspondence to: Dr F Byrne, Amgen Inc, One Amgen Center Drive, Mail Stop 29-1-B, Thousand Oaks, California 91320, USA; fbyrne@amgen.com

Revised version received 23 April 2004
Accepted for publication 9 June 2004

Bone loss and osteoporosis have long been recognised as long term sequelae of inflammatory bowel diseases (IBD).^{1–2} Many studies have shown a direct link between inflammatory disease and bone loss, both in animal models^{3–5} and human disease.^{6–8} However, the relative contributions of inflammation and/or malabsorption to bone mineral density (BMD) loss is still unclear.^{9–11} Although various therapies have been tried to specifically treat the disease associated bone loss,^{12–14} there is still an acute need for effective targeted therapy for this extraintestinal manifestation of the disease.

Osteoprotegerin (OPG) is a soluble decoy receptor that can inhibit the differentiation, survival, and function of osteoclasts by binding and neutralising receptor activator of nuclear factor κ B ligand (RANKL), thus preventing the latter from stimulating receptor activator of nuclear factor κ B (RANK) on osteoclasts.^{15–16} OPG knockout mice develop severe early onset osteoporosis^{17–18} whereas OPG transgenic mice develop osteopetrosis.¹⁹ Administration of recombinant OPG has been shown to be effective at preventing bone loss in animal models of arthritis,^{20–22} bone cancer metastasis,²³ and oestrogen deficiency associated with ovariectomy.

We sought to determine whether the CD4⁺CD45RB^{Hi} T cell transfer model of IBD was associated with osteopenia. Secondly, we aimed to determine whether recombinant

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.

MATERIALS AND METHODS

Mice and reagents

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.²⁴

Induction of IBD-like disease

Disease was induced by intraperitoneal injection of 4×10^5 sorted CD4⁺CD45RB^{Hi} or CD4⁺CD45RB^{Lo} (control) T cells

Abbreviations: ALP, alkaline phosphatase; BMD, bone mineral density; IBD, inflammatory bowel disease; OPG, osteoprotegerin; PTH, parathyroid hormone; pQCT, peripheral quantitative computer tomography; RANK(L), receptor activator of nuclear factor κ B (ligand); SAA, serum amyloid A; TRAP, tartrate resistant acid phosphatase; TNF- α , tumour necrosis factor α

Figure 1 Transfer of CD4⁺CD45RB^{Hi} T cells to *scid/scid* mice caused significant inflammatory cell infiltrate and histopathological damage to the large intestine (B) compared with transfer of CD4⁺CD45RB^{Lo} T cells which had no significant effect on the histopathology of the large intestine of immunodeficient mice (A). Administration of Fc-osteoprotegerin (OPG) has no significant effect on the large intestine of CD4⁺CD45RB^{Lo} (C) or CD4⁺CD45RB^{Hi} (D) mice. (A) CD4⁺CD45RB^{Lo} cells treated with 3.4 mg/kg of Fc three times per week starting on day 0. (B) CD4⁺CD45RB^{Hi} cells treated with 3.4 mg/kg of Fc three times per week starting on day 0. (C) CD4⁺CD45RB^{Lo} cells treated with 5.0 mg/kg of Fc-OPG control three times per week starting on day 0. (D) CD4⁺CD45RB^{Hi} cells treated with 5.0 mg/kg of Fc-OPG three times per week starting on day 0. A representative haematoxylin and eosin stained biopsy from the middle of the large intestine is shown (see materials and methods) in each case. Note the significant mixed cell type inflammatory infiltrate (*), mucin depletion, and crypt abscessation present in the mucosa of the diseased mice injected with CD4⁺CD45RB^{Hi} cells.

from CB6F1 mice into immunodeficient C.B.17 *scid/scid* mice.²⁵ 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⁺CD45RB^{Hi}) 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.¹⁹ The thoracic vertebrae, as one unit, were fixed and prepared for histological examination as above.¹⁹ 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.²⁶ 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 0–5.²⁵ 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

Figure 2 Transfer of CD4⁺CD45RB^{Hi} T cells (RB Hi) to *scid/scid* mice caused significant inflammation and histopathological damage to the large intestine (LI) of immunodeficient mice whereas transfer of CD4⁺CD45RB^{Lo} T cells (RB Lo) had no significant effect on the histopathology of the large intestine. Administration of Fc-osteoprotegerin (OPG) had no significant effect on the large intestine of CD4⁺CD45RB^{Lo} or CD4⁺CD45RB^{Hi} mice. Disease was induced by injection of 4×10^5 sorted CD4⁺CD45RB^{Hi} T cells into *scid/scid* mice. The negative control mice received 4×10^5 CD4⁺CD45RB^{Lo} 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⁺CD45RB^{Hi} groups and $n = 4$ for the CD4⁺CD45RB^{Lo} groups. Statistical significance versus the CD4⁺CD45RB^{Lo} group treated with Fc was calculated by the probability associated with the unpaired heteroschedastic Student's *t* test: *** $p < 0.001$.

primarily on inflammatory infiltrate.²³ All samples were scored by a pathologist in a blinded fashion.

Serum amyloid A, complete blood counts, and clinical chemistries

Serum amyloid (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 an H1E counter (Technicon, Terrytown, 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 tartarate 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 μ m) of the paraffin embedded, formalin fixed, decalcified tibia were

Figure 3 Transfer of CD4⁺CD45RB^{Hi} T cells to *scid/scid* mice caused an obvious decrease in the radio-opacity of the distal femur (B) compared with transfer of CD4⁺CD45RB^{Lo} T cells (A). Prophylactic administration of Fc-osteoprotegerin (OPG) to the CD4⁺CD45RB^{Lo} 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⁺CD45RB^{Hi} mice (D). Disease was induced by injection of 4×10^5 sorted CD4⁺CD45RB^{Hi} T cells into *scid/scid* mice. Negative control mice received 4×10^5 CD4⁺CD45RB^{Lo} 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. Excised femurs were fixed in 70% ethanol, radiographed with a Faxitron x ray system (Model 43855A Faxitron; X-ray Corp, Buffalo Grove, Illinois, USA) with an exposure time of 49 seconds, and magnified 645% with scanning software (DeskScan II v.2.8; Hewlett Packard, Palo Alto, California, USA).

processed by standard in situ hybridisation techniques, as previously described,²⁷ and hybridised overnight with 2×10^6 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 χ^2 analysis.

RESULTS

Transfer of CD4⁺CD45RB^{Hi} T cells to immunodeficient mice causes body weight loss and systemic and colonic inflammation, which are unaffected by treatment with Fc-OPG

Transfer of CD4⁺CD45RB^{Hi} T cells to immunodeficient mice caused significant progressive body weight loss whereas transfer of CD4⁺CD45RB^{Lo} T cells had no significant effect on body weight ($p < 0.0001$) (data not shown). Similar results were seen with elevated levels of SAA or other disease associated haematological abnormalities, including an elevated white blood cell count, depressed red blood cell count,

Figure 4 Transfer of CD4⁺CD45RB^{Hi} 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⁺CD45RB^{Lo} T cells (RB Lo) (A). CD4⁺CD45RB^{Hi} T cells also caused a decrease in total BMD of the L5 lumbar vertebra (B). Administration of Fc-osteoprotegerin (OPG) to CD4⁺CD45RB^{Lo} 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⁺CD45RB^{Hi} 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 4×10^5 sorted CD4⁺CD45RB^{Hi} T cells into *scid/scid* mice. Negative control mice received 4×10^5 CD4⁺CD45RB^{Lo} 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. Bone density was analysed by peripheral quantitative computer tomography of two 1.25 mm cross sections of the proximal end of the tibia or the L5 vertebrae proximal to the iliac crest. In all cases results represent mean (SEM) values, with $n=7-8$ for the CD4⁺CD45RB^{Hi} groups and $n=4$ for the CD4⁺CD45RB^{Lo} groups. Statistical significance versus the CD4⁺CD45RB^{Lo} group treated with Fc was calculated by the probability associated with the unpaired heteroschedastic Student's *t* test: * $p<0.05$, *** $p<0.001$.

and depressed haemoglobin and haematocrit (data not shown). The colon is the primary site of inflammation in this model and fig 1 clearly shows that transfer of CD4⁺CD45RB^{Hi} T cells to immunodeficient mice caused inflammatory cell infiltrate and histopathological damage to the large intestine (fig 1B), compared with transfer of CD4⁺CD45RB^{Lo} T cells which had no effect (fig 1A). Administration of Fc-OPG had no significant further effect on the large intestine of CD4⁺CD45RB^{Lo} (fig 1C) or CD4⁺CD45RB^{Hi} (fig 1D) mice. Quantification and statistical analysis of all of the experimental groups confirmed this observation (CD4⁺CD45RB^{Lo} *v* CD4⁺CD45RB^{Hi}, $p<0.0001$) (see fig 2) and also showed that therapeutic administration of Fc-OPG—after the mice had lost 10% of their original body

Figure 5 Transfer of CD4⁺CD45RB^{Hi} 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⁺CD45RB^{Lo} T cells (RB Lo). Administration of Fc-osteoprotegerin (OPG) to control CD4⁺CD45RB^{Lo} 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⁺CD45RB^{Hi} mice. Disease was induced by injection of 4×10^5 sorted CD4⁺CD45RB^{Hi} T cells into *scid/scid* mice. Negative control mice received 4×10^5 CD4⁺CD45RB^{Lo} 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⁺CD45RB^{Hi} groups and $n=4$ for the CD4⁺CD45RB^{Lo} groups. The numbers of osteoclasts and osteoblasts were determined by bone histomorphometry, as described in the materials and methods. Statistical significance versus the CD4⁺CD45RB^{Lo} group treated with Fc was calculated by the probability associated with the unpaired heteroschedastic Student's *t* test: * $p<0.05$.

weight—had no significant effect on the histopathological damage to the large intestine caused by transfer of the CD4⁺CD45RB^{Hi} T cells.

Hence all of the primary parameters associated with IBD-like disease were unchanged with either prophylactic or therapeutic treatment with Fc-OPG and were within normal values. The lack of any effect of administration of recombinant Fc-OPG on any of the inflammation end points associated with this disease model of IBD is consistent with other animal models of autoimmune disease,^{20,22} as well as cancer²³ and hypercalcaemia.²⁴

CD4⁺CD45RB^{Hi} 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⁺CD45RB^{Hi} T cells to immunodeficient mice caused a visible decrease in the radio-opacity

Figure 6 Transfer of CD4⁺CD45RB^{Hi} T cells to *scid/scid* mice caused significant inflammatory cell infiltrate (B) and an increase in the numbers of osteoclasts (B, inset) compared with transfer of CD4⁺CD45RB^{Lo} T cells which had no significant effect on the composition of normal bone marrow (A). Administration of Fc-osteoprotegerin (OPG) caused increased amounts of trabecular bone and thickening of the cortical bone in CD4⁺CD45RB^{Lo} mice (C) and had a similar effect in diseased CD4⁺CD45RB^{Hi} mice, despite the presence of the inflammatory infiltrate (D), by greatly reducing the numbers of osteoclasts (C and D insets). (A) CD4⁺CD45RB^{Lo} cells treated with 3.4 mg/kg of Fc three times per week starting on day 0. (B) CD4⁺CD45RB^{Hi} cells treated with 3.4 mg/kg of Fc three times per week starting on day 0. (C) CD4⁺CD45RB^{Lo} cells treated with 5.0 mg/kg of Fc-OPG control three times per week starting on day 0. (D) CD4⁺CD45RB^{Hi} cells treated with 5.0 mg/kg of Fc-OPG three times per week starting on day 0. Disease was induced by injection of 4×10^5 sorted CD4⁺CD45RB^{Hi} T cells into *scid/scid* mice. Negative control mice received 4×10^5 CD4⁺CD45RB^{Lo} 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.

of the distal femur (fig 3B) compared with that observed with transfer of CD45RB^{Lo} T cells (fig 3A). Prophylactic administration of Fc-OPG to control CD4⁺CD45RB^{Lo} 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^{Hi} mice (fig 3D). We quantified changes in BMD via pQCT analysis of the tibia. Figure 4 demonstrates that transfer of CD4⁺CD45RB^{Hi} 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^{Lo} T cells ($p < 0.0001$) (fig 4A). CD4⁺CD45RB^{Hi} T cells also caused a decrease in total BMD of the L5 lumbar vertebra (fig 4B). Administration of Fc-OPG to control CD4⁺CD45RB^{Lo} 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^{Hi} 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^{Lo} mice 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^{Hi} 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.^{20, 22} Figure 5 shows that the transfer of CD4⁺CD45RB^{Hi} 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^{Lo} T cells. Administration of Fc-OPG to control CD4⁺CD45RB^{Lo} 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^{Hi} mice. This observation is consistent with previous studies,²⁰ and the simultaneous elimination of osteoblasts is thought to result from the normal coupling of osteoblast and osteoclast metabolism.

CD4⁺CD45RB^{Hi} 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

Figure 7 Transfer of CD4⁺CD45RB^{Hi} T cells (RB Hi) to *scid/scid* mice caused significant inflammatory cell infiltrate in the bone marrow of *scid/scid* mice compared with transfer of CD4⁺CD45RB^{Lo} T cells (RB Lo). The effect was equally pronounced in the tibia/femur and thoracic vertebrae. Administration of Fc-osteoprotegerin (OPG) had no significant effect on the infiltrate present in CD4⁺CD45RB^{Lo} mice or diseased CD4⁺CD45RB^{Hi} mice. Disease was induced by injection of 4×10^5 sorted CD4⁺CD45RB^{Hi} T cells into *scid/scid* mice. Negative control mice received 4×10^5 CD4⁺CD45RB^{Lo} 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 bones were assessed for extent of inflammation on a scale of 0–5 (0 = normal myelopoiesis, 5 = massive myeloid hyperplasia) by a pathologist in a blinded fashion. In all cases results represent mean (SEM) values with $n = 7-8$ for the CD4⁺CD45RB^{Hi} groups and $n = 4$ for the CD4⁺CD45RB^{Lo} groups. Statistical significance versus the vehicle control was calculated by the probability associated with the unpaired heteroschedastic Student's *t* test: * $p < 0.05$, *** $p < 0.001$.

rapid compensatory increase in serum PTH. We therefore measured serum PTH levels mid way through the study as well as at necropsy to look for indirect evidence of impaired calcium absorption. Transfer of CD4⁺CD45RB^{Hi} T cells to immunodeficient mice caused a highly significant decrease in levels of circulating PTH (2.3 (1.5) pg/ml) at the time of necropsy compared with transfer of CD4⁺CD45RB^{Lo} T cells (27.4 (2.2) pg/ml) ($p < 0.0001$). Administration of Fc-OPG to control CD4⁺CD45RB^{Lo} mice decreased levels of circulating PTH (8.8 (7.0) pg/ml) but there was considerable variation in the reduction ($p = 0.0495$). Prophylactic or therapeutic administration of Fc-OPG partially normalised levels of circulating PTH in diseased CD4⁺CD45RB^{Hi} mice relative to CD4⁺CD45RB^{Lo} mice (prophylactic 15.5 (4.6) pg/ml, $p = 0.0385$; therapeutic 15.1 (6.9) pg/ml, $p = 0.1136$).

In a separate experiment, serum PTH was also analysed at the 10% body weight loss time point (approximately day 25,

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⁺CD45RB^{Lo} control mice ($p = 0.6406$) (data not shown).

CD4⁺CD45RB^{Hi} 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⁺CD45RB^{Hi} cells had similar levels of serum calcium to mice injected with CD4⁺CD45RB^{Lo} 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⁺CD45RB^{Hi} cells did not cause a change in levels of serum phosphorous (9.2 (0.4) g/dl) compared with control CD4⁺CD45RB^{Lo} cells (9.0 (0.5) g/dl; $p = 0.7475$). Diseased CD4⁺CD45RB^{Hi} mice treated prophylactically with Fc-OPG had significantly lower serum phosphorus (8.0 (0.4) g/dl; $p = 0.0561$), as did diseased CD4⁺CD45RB^{Hi} mice treated with therapeutic Fc-OPG (7.6 (0.5) 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⁺CD45RB^{Hi} cells caused a significant rise in levels of serum TRAP (6.00 (0.45) U/l) compared with transfer of CD4⁺CD45RB^{Lo} cells (3.48 (0.3) U/l; $p < 0.001$). CD4⁺CD45RB^{Lo} mice treated with Fc-OPG did not have significantly changed levels of TRAP (5.18 (2.13) U/l; $p = 0.4267$). Levels of TRAP in diseased mice treated prophylactically with Fc-OPG (4.24 (0.85)) were normalised and were not significantly different to CD4⁺CD45RB^{Lo} 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)) 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.^{28–29}

CD4⁺CD45RB^{Hi} mice had significantly lower levels of alkaline phosphatase (ALP) (54.9 (3.5) U/l) compared with control CD4⁺CD45RB^{Lo} mice (83.3 (3.8) U/l; $p < 0.0001$). Control mice treated with Fc-OPG also had significantly depressed levels of ALP (52.3 (3.6) U/l; $p < 0.0001$). Levels of ALP in diseased mice treated with prophylactic Fc-OPG were depressed even further (34.6 (1.3) U/l; $p = 0.0002$ relative to control CD4⁺CD45RB^{Lo} mice), and similarly in therapeutic Fc-OPG treatment (40.6 (4.6) U/l; $p = 0.0002$ relative to control CD4⁺CD45RB^{Lo} 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.^{30–32}

CD4⁺CD45RB^{Hi} 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.^{20–23} Hence we examined whether there was significant inflammatory cell infiltrate in the bones of diseased mice in this model of IBD. Transfer of CD4⁺CD45RB^{Hi} 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

Figure 8 Transfer of CD4⁺CD45RB^{Hi} 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⁺CD45RB^{Lo} 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⁺CD45RB^{Lo} mice (C) or CD4⁺CD45RB^{Hi} mice (D). (A) CD4⁺CD45RB^{Lo} cells treated with 3.4 mg/kg of Fc three times per week starting on day 0. (B) CD4⁺CD45RB^{Hi} cells treated with 3.4 mg/kg of Fc three times per week starting on day 0. (C) CD4⁺CD45RB^{Lo} cells treated with 5.0 mg/kg of Fc-OPG control three times per week starting on day 0. (D) CD4⁺CD45RB^{Hi} cells treated with 5.0 mg/kg of Fc-OPG three times per week starting on day 0. Disease was induced by injection of 4×10^5 sorted CD4⁺CD45RB^{Hi} T cells into *scid/scid* mice. Negative control mice received 4×10^5 non-pathogenic CD4⁺CD45RB^{Lo} 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.

(fig 6B, main picture) compared with transfer of non-pathogenic control CD4⁺CD45RB^{Lo} T cells which had no significant effect. This T cell transfer had no significant effect on the composition of normal bone marrow (fig 6A). Administration of Fc-OPG caused increased amounts of trabecular bone and thickening of the cortical bone in control CD4⁺CD45RB^{Lo} mice (fig 6C). Fc-OPG had a similar effect in diseased CD4⁺CD45RB^{Hi} mice, despite the presence of the inflammatory infiltrate (fig 6D), by greatly reducing the numbers of osteoclasts (fig 6C, 6D inserts). Quantification of the inflammatory infiltrate and statistical analysis of all of the experimental groups confirmed this observation (CD4⁺CD45RB^{Hi} v CD4⁺CD45RB^{Lo}; $p < 0.0001$) (fig 7) and also showed that therapeutic administration of Fc-OPG—after the mice had lost 10% of their original body weight—had no further significant effect on the inflammatory infiltrate of the bone caused by transfer of CD4⁺CD45RB^{Hi} T cells. It is noteworthy that the inflammatory infiltrate of the bone was equally pronounced in both the tibia/femur and thoracic vertebrae. The increased numbers of osteoclasts noted in diseased mice and the decreased numbers evident in Fc-OPG treated mice is consistent with the histomorphometric analysis and levels of TRAP and ALP previously described.

Recent work has further defined the mechanism by which inflammation leads to bone loss, specifically the importance of TNF- α in inducing RANKL and subsequent osteoclastogenesis.^{10 21 33} In an attempt to establish the actions of the inflammatory cells in bone marrow, we examined levels of TNF- α mRNA by in situ hybridisation. Transfer of control

CD4⁺CD45RB^{Hi} T cells to *scid/scid* mice caused significant upregulation of TNF- α mRNA (fig 8B) whereas transfer of CD4⁺CD45RB^{Lo} T cells had no significant effect on expression of TNF- α mRNA in the shaft of the tibia (fig 8A). Administration of Fc-OPG to either control CD4⁺CD45RB^{Lo} mice (fig 8C) or diseased CD4⁺CD45RB^{Hi} mice (fig 8D) had no significant further effect on the observed levels of TNF- α mRNA. Quantification of the inflammatory infiltrate and statistical analysis of all of the experimental groups confirmed this observation in that CD4⁺CD45RB^{Hi} mice had detectable levels of TNF- α mRNA in 6/9 mice and treatment with Fc-OPG, in either prophylactic or therapeutic regimens, did not significantly change this observed penetrance (5/7 and 6/9, respectively). However, this TNF- α infiltrate was significantly different from control CD4⁺CD45RB^{Lo} mice by χ^2 analysis ($p = 0.0035$) which, in contrast, had detectable TNF- α mRNA in 1/6 mice in both the Fc and Fc-OPG treated groups.

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,^{34–35} with occurrence in the ulcerative colitis population being less well established.^{11–36} 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 premorbid course and more extraintestinal manifestations.³⁷ We chose this mouse model of IBD to be representative of the Crohn's disease variant of IBD³⁸ 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.^{11–34–39}

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.^{9–11–40} 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,^{41–44} 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.^{33–36–45–48} It has further been shown that RANKL can act to maintain inflammation.⁴⁹ Although the reported data in human studies are 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.^{11–36} 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 supplementation¹² or glucagon-like peptide 2,¹³ to the use of bisphosphonates¹⁴ 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.^{20–50–52} 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 osteopenia 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.²⁰

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.

ACKNOWLEDGEMENTS

We are grateful to Jorge Arias and Jenny Smith of Amgen Inc., Department of Laboratory Animal Resources, for excellent technical assistance and advice, and to Yan Cheng, Annie Luo, and Darlene Kratavil of Amgen Inc., Department of Pathology and Pharmacology, for excellent sample histology processing. We are also grateful to Tom Graves of Amgen Inc., Department of Biostatistics, for assistance with statistical analysis.

Authors' affiliations

F R Byrne, H L Brown, S A Flores, M Fiorino, S L Yin, G Senaldi, Department of Inflammation, Amgen Inc., Thousand Oaks, California, USA
S Morony, K Warmington, Z Geng, D Hill, V Porkess, P J Kostenuik, Department of Metabolic Disorders, Amgen Inc., Thousand Oaks, California, USA
D Duryea, J K Pretorius, S Adamu, D M Danilenko, I Sarosi, D L Lacey, Department of Pathology, Amgen Inc., Thousand Oaks, California, USA
R Manuokian, Department of Pre-clinical and Protein Therapeutics, Amgen Inc., Thousand Oaks, California, USA

Conflict of interest: None declared.

REFERENCES

- 1 **Andreassen H,** Rungby J, Dahlerup JF, *et al.* Inflammatory bowel disease and osteoporosis. *Scand J Gastroenterol* 1997;**32**:1247–55.
- 2 **Silvennoinen JA,** Karttunen TJ, Niemela SE, *et al.* A controlled study of bone mineral density in patients with inflammatory bowel disease. *Gut* 1995;**37**:71–6.
- 3 **Campagnuolo G,** Bolon B, Feige U. Kinetics of bone protection by recombinant osteoprotegerin therapy in Lewis rats with adjuvant arthritis. *Arthritis Rheum* 2002;**46**:1926–36.
- 4 **Hanyu T,** Chotanaphuti T, Arai K, *et al.* Histomorphometric assessment of bone changes in rats with type II collagen-induced arthritis. *Bone* 1999;**24**:485–90.
- 5 **Kong YY,** Feige U, Sarosi I, *et al.* Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. *Nature* 1999;**402**:304–9.

- 6 Hiramata T, Danks L, Sabokbar A, et al. Osteoclast formation and activity in the pathogenesis of osteoporosis in rheumatoid arthritis. *Rheumatology* 2002;**41**:1232-9.
- 7 Dolan AL, Moniz C, Abraha H, et al. Does active treatment of rheumatoid arthritis limit disease-associated bone loss? *Rheumatology* 2002;**41**:1047-51.
- 8 Haugeberg G, Orstavik RE, Uhlig T, et al. Bone loss in patients with rheumatoid arthritis: results from a population-based cohort of 366 patients followed up for two years. *Arthritis Rheum* 2002;**46**:1720-8.
- 9 Jahnsen J, Falch JA, Mowinkel P, et al. Vitamin D status, parathyroid hormone and bone mineral density in patients with inflammatory bowel disease. *Scand J Gastroenterol* 2002;**37**:192-9.
- 10 Komine M, Kukita A, Kukita T, et al. Tumor necrosis factor- α cooperates with receptor activator of nuclear factor kappaB ligand in generation of osteoclasts in stromal cell-depleted rat bone marrow cell culture. *Bone* 2001;**28**:474-83.
- 11 Lee SH, Kim HJ, Yang SK, et al. Decreased trabecular bone mineral density in newly diagnosed inflammatory bowel disease patients in Korea. *J Gastroenterol Hepatol* 2000;**15**:512-18.
- 12 Bernstein CN, Seeger LL, Anton PA, et al. A randomized, placebo-controlled trial of calcium supplementation for decreased bone density in corticosteroid-using patients with inflammatory bowel disease: a pilot study. *Aliment Pharmacol Ther* 1996;**10**:777-86.
- 13 Haderslev KV, Jeppesen PB, Hartmann B, et al. Short-term administration of glucagon-like peptide-2. Effects on bone mineral density and markers of bone turnover in short-bowel patients with no colon. *Scand J Gastroenterol* 2002;**37**:392-8.
- 14 Haderslev KV, Tjellesen L, Sorensen HA, et al. Alendronate increases lumbar spine bone mineral density in patients with Crohn's disease. *Gastroenterology* 2000;**119**:639-46.
- 15 Burgess TL, Qian Y, Kaufman S, et al. The ligand for osteoprotegerin (OPGL) directly activates mature osteoclasts. *J Cell Biol* 1999;**145**:527-38.
- 16 Hsu H, Lacey DL, Dunstan CR, et al. Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. *Proc Natl Acad Sci U S A* 1999;**96**:3540-5.
- 17 Bucay N, Sarosi I, Dunstan CR, et al. Osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. *Genes Dev* 1998;**12**:1260-8.
- 18 Lacey DL, Tan HL, Lu J, et al. Osteoprotegerin ligand modulates murine osteoclast survival in vitro and in vivo. *Am J Pathol* 2000;**157**:435-48.
- 19 Simonet WS, Lacey DL, Dunstan CR, et al. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell* 1997;**89**:309-19.
- 20 Campagnuolo G, Bolon B, Feige U. Kinetics of bone protection by recombinant osteoprotegerin therapy in Lewis rats with adjuvant arthritis. *Arthritis Rheum* 2002;**46**:1926-36.
- 21 Kong YY, Feige U, Sarosi I, et al. Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. *Nature* 1999;**402**:304-9.
- 22 Romas E, Sims NA, Hards DK, et al. Osteoprotegerin reduces osteoclast numbers and prevents bone erosion in collagen-induced arthritis. *Am J Pathol* 2002;**161**:1419-27.
- 23 Morony S, Capparelli C, Sarosi I, et al. Osteoprotegerin inhibits osteolysis and decreases skeletal tumor burden in syngeneic and nude mouse models of experimental bone metastasis. *Cancer Res* 2001;**61**:4432-6.
- 24 Morony S, Capparelli C, Lee R, et al. A chimeric form of osteoprotegerin inhibits hypercalcemia and bone resorption induced by IL-1 β , TNF- α , PTH, PTHrP, and 1, 25(OH) $_2$ D $_3$. *J Bone Miner Res* 1999;**14**:1478-85.
- 25 Byrne FR, Farrell CL, Aranda R, et al. rHuKGF ameliorates symptoms in DSS and CD4 $^{+}$ CD45RB(Hi) T cell transfer mouse models of inflammatory bowel disease. *Am J Physiol Gastrointest Liver Physiol* 2002;**282**:G690-701.
- 26 Kostenuik PJ, Capparelli C, Morony S, et al. OPG and PTH-(1-34) have additive effects on bone density and mechanical strength in ovariectomized rats. *Endocrinology* 2001;**142**:4295-304.
- 27 Wilcox JN. Fundamental principles of in situ hybridization. *J Histochem Cytochem* 1993;**41**:1725-33.
- 28 Kurata K, Uemura T, Nemoto A, et al. Mechanical strain effect on bone-resorbing activity and messenger RNA expressions of marker enzymes in isolated osteoclast culture. *J Bone Miner Res* 2001;**16**:722-30.
- 29 Sasaki N, Kusano E, Ando Y, et al. Changes in osteoprotegerin and markers of bone metabolism during glucocorticoid treatment in patients with chronic glomerulonephritis. *Bone* 2002;**30**:853-8.
- 30 Ueda M, Inaba M, Okuno S, et al. Clinical usefulness of the serum N-terminal propeptide of type I collagen as a marker of bone formation in hemodialysis patients. *Am J Kidney Dis* 2002;**40**:802-9.
- 31 Yan T, Wergedal J, Zhou Y, et al. Inhibition of human osteoblast marker gene expression by retinoids is mediated in part by insulin-like growth factor binding protein-6. *Growth Hormone IGF Res* 2001;**11**:368-77.
- 32 Bonucci E, Nanci A. Alkaline phosphatase and tartrate-resistant acid phosphatase in osteoblasts of normal and pathologic bone. *Ital J Anat Embryol* 2001;**106**(suppl 1):129-33.
- 33 Zhang YH, Heulsmann A, Tondravi MM, et al. Tumor necrosis factor- α (TNF) stimulates RANKL-induced osteoclastogenesis via coupling of TNF type 1 receptor and RANK signaling pathways. *J Biol Chem* 2001;**276**:563-8.
- 34 Bernstein CN, Blanchard JF, Leslie W, et al. The incidence of fracture among patients with inflammatory bowel disease—A population-based cohort study. *Ann Intern Med* 2000;**133**:795-9.
- 35 Buchman AL. Bones and Crohn's: problems and solutions. *Inflamm Bowel Dis* 1999;**5**:212-27.
- 36 Ardizzone S, Bollani S, Bettica P, et al. Altered bone metabolism in inflammatory bowel disease: there is a difference between Crohn's disease and ulcerative colitis. *J Intern Med* 2000;**247**:63-70.
- 37 Veloso FT, Carvalho J, Magro F. Immune-related systemic manifestations of inflammatory bowel disease—a prospective study of 792 patients. *J Clin Gastroenterol* 1996;**23**:29-34.
- 38 De Winter H, Cheroutre H, Kronenberg M. Mucosal immunity and inflammation II. The yin and yang of T cells in intestinal inflammation: pathogenic and protective roles in a mouse colitis model. *Am J Physiol Gastrointest Liver Physiol* 1999;**39**:G1317-21.
- 39 Schoon EJ, Blok BM, Geerling BJ, et al. Bone mineral density in patients with recently diagnosed inflammatory bowel disease. *Gastroenterology* 2000;**119**:1203-1.
- 40 Schoon EJ, Muller MCA, Vermeer C, et al. Low serum and bone vitamin K status in patients with longstanding Crohn's disease: Another pathogenic factor of osteoporosis in Crohn's disease? *Gut* 2001;**48**:473-7.
- 41 Steiner PD, Forrer R, Kneissel M, et al. Influence of a low calcium and phosphorus diet on the anabolic effect of human parathyroid hormone (1-38) in female rats. *Bone* 2001;**29**:344-51.
- 42 Wolinsky I, Guggenheim K. Effect of low calcium diet on bone and calcium metabolism in rats and mice—a differential species response. *Comp Biochem Physiol A* 1974;**49**:183-95.
- 43 Bourrin S, Toromanoff A, Ammann P, et al. Dietary protein deficiency induces osteoporosis in aged male rats. *J Bone Miner Res* 2000;**15**:1555-63.
- 44 Talbot SM, Rothkopf MM, Shapses SA. Dietary restriction of energy and calcium alters bone turnover and density in younger and older female rats. *J Nutr* 1998;**128**:640-5.
- 45 Fox SW, Fuller K, Bayley KE, et al. TGF- β 1 and IFN- γ direct macrophage activation by TNF- α to osteoclastic or cytotoxic phenotype. *J Immunol* 2000;**165**:4957-63.
- 46 Kotake S, Udagawa N, Hakoda M, et al. Activated human T cells directly induce osteoclastogenesis from human monocytes: possible role of T cells in bone destruction in rheumatoid arthritis patients. *Arthritis Rheum* 2001;**44**:1003-12.
- 47 Theill LE, Boyle WJ, Penninger JM. RANK-L and RANK: T cells, bone loss, and mammalian evolution. *Annu Rev Immunol* 2002;**20**:795-823.
- 48 Weitzmann MN, Cenci S, Rifas L, et al. T cell activation induces human osteoclast formation via receptor activator of nuclear factor kappaB ligand-dependent and -independent mechanisms. *J Bone Miner Res* 2001;**16**:328-37.
- 49 Josien R, Li HL, Ingulli E, et al. TRANCE, a tumor necrosis factor family member, enhances the longevity and adjuvant properties of dendritic cells in vivo. *J Exp Med* 2000;**191**:495-501.
- 50 Bateman TA, Dunstan CR, Ferguson VL, et al. Osteoprotegerin mitigates tail suspension-induced osteopenia. *Bone* 2000;**26**:443-9.
- 51 Bolon B, Carter C, Daris M, et al. Adenoviral delivery of osteoprotegerin ameliorates bone resorption in a mouse ovariectomy model of osteoporosis. *Mol Ther J Am Soc Gene Ther* 2001;**3**:197-205.
- 52 Capparelli C, Kostenuik PJ, Morony S, et al. Osteoprotegerin prevents and reverses hypercalcemia in a murine model of humoral hypercalcemia of malignancy. *Cancer Res* 2000;**60**:783-7.

PostScript

LETTERS

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 aetiopathogenesis 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 stimulation¹ although recent data have questioned the functional effect of this variant.²

We therefore note with interest the article of 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. Torok *et al* examined the presence of both the Asp299Gly and Thr399Ile polymorphisms in a smaller German IBD population.³ 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.⁴

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 Lorenz¹ 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.⁵

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%).^{6,7} 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 508 mutation in cystic fibrosis. Lower CARD15 (NOD2) frequencies in CD have been reported from Scotland⁴ and Finland⁸ and are absent in a small Icelandic population.⁹

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.

I D R Arnott, G-T Ho, E R Nimmo, J Satsangi
Western General Hospital, Edinburgh, Scotland, UK

Correspondence to: Dr I D R Arnott, Western General Hospital, Crewe Rd, Edinburgh EH4 2XU, Scotland, UK; ian.arnott@doctors.net.uk

Conflict of interest: None declared.

References

- 1 Arbour NC, Lorenz E. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. *Nat Genet* 2000;25:187–91.
- 2 Erridge C, Stewart J, Poxton IR. Monocytes heterozygous for the Asp299Gly and Thr399Ile mutations in the Toll-like receptor 4 gene show no deficit in lipopolysaccharide signalling. *J Exp Med* 2003;197:1787–91.
- 3 Torok HP, Glas J, Tonenchi L, *et al*. Polymorphisms of the lipopolysaccharide-signaling complex in inflammatory bowel disease: association of a mutation in the Toll-like receptor 4 gene with ulcerative colitis. *Clin Immunol* 2004;112:85–91.
- 4 Arnott ID, Nimmo ER. NOD2/CARD15, TLR4 and CD14 mutations in Scottish and Irish Crohn's disease patients: evidence for genetic heterogeneity within Europe? *Genes Immun* 2004;5:417–25.
- 5 Okayama N, Fujimura K. Simple genotype analysis of the Asp299Gly polymorphism of the Toll-like receptor-4 gene that is associated with lipopolysaccharide hyporesponsiveness. *J Clin Lab Anal* 2002;16:56–8.
- 6 Marsh S, McLeod HL. Crohn's disease: ethnic variation in CARD15 genotypes. *Gut* 2003;52:770.
- 7 Bonen DK, Cho JH. The genetics of inflammatory bowel disease. *Gastroenterology* 2003;124:521–36.
- 8 Helio T, Halme L. CARD15/NOD2 gene variants are associated with familiarly occurring and complicated forms of Crohn's disease. *Gut* 2003;52:558–62.
- 9 Thjodleifsson B, Sigthorsson G. Subclinical intestinal inflammation: an inherited abnormality in Crohn's disease relatives? *Gastroenterology* 2003;124:1728.

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 reporting 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

	Patient numbers (allele frequencies)			Crohn's disease genotype frequencies		
	CD	UC	HC	Wild-type	Heterozygous	Homozygous
Leuven	334 (11.0%)	163 (10.0%)	139 (5.0%)	79.3	19.5	1.2
Munich	102 (7.0%)	98 (9.0%)	145 (4.0%)	85.0	15.0	0
Edinburgh	234 (10.3%)	246 (6.8%)	189 (8.8%)	79.5	19.7	0.8

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).

disease to another Crohn's disease) but also, possibly, from one population to another (or from one ethnic background to another). In fact, the relative proportion of polymorphisms in susceptible genes that influence a specific phenotype may vary depending on the ethnic background. As highlighted by the Edinburgh group, the population attributable risk for NOD2 variants is significantly lower in Scotland and Ireland than in the rest of Europe or the USA, suggesting a much lower contribution of NOD2 variants (or perhaps of pattern recognition receptors (PRRs)) in Crohn's disease.¹ Interestingly, this is reinforced by their recent observation of a negative association between Crohn's disease and the TLR4 Asp299Gly polymorphism. High resolution haplotype mapping of TLRs and signalling molecules should allow us to discriminate the relative influence of these PRRs in Crohn's disease and ulcerative colitis across the world.

D Franchimont

Erasmus University Hospital, Brussels, Belgium

S Vermeire

University Hospital Gasthuisberg, Leuven, Belgium

J Devere

Erasmus University Hospital, Brussels, Belgium

P J Rutgeerts

University Hospital Gasthuisberg, Leuven, Belgium

Correspondence to: Dr D Franchimont, Erasmus University Hospital, Lennik St, 808 Brussels, Belgium; denis.franchimont@ulb.ac.be

Conflict of interest: None declared.

Reference

- 1 **Arnot ID, Nimmo ER.** NOD2/CARD15, TLR4 and CD14 mutations in Scottish and Irish Crohn's disease patients: evidence for genetic heterogeneity within Europe? *Genes Immun* 2004;**5**:417–25.

Portopulmonary hypertension in cirrhosis: the pathogenetic challenge

We would like to thank Pellicelli (*Gut* 2004;**53**:1721) for his interest in our paper on portopulmonary hypertension in cirrhosis with refractory ascites (*Gut* 2003;**52**:1355–62).

We acknowledge that our proposal suggesting the role of endothelin 1 (ET-1) in the pathogenesis of portopulmonary hypertension is based on a small number of patients. However, the strong association between the elevated ET-1 levels in the pulmonary artery and the presence of portopulmonary hypertension in these patients would suggest that this is indeed the case. The confirmatory test would be a reduction in pulmonary pressure following blockade of endothelin receptors in the pulmonary circulation. However, this has not been reported to date. We also acknowledge that we did not measure the gradient of ET-1 across the pulmonary vascular bed, which is an important site for both production and clearance of ET-1. The fact that systemic arterial endothelin levels in patients with advanced cirrhosis are similar to those in control subjects¹ would suggest that there is no net production of ET-1 in the pulmonary circulation. Rather, the high levels of ET-1

delivered to the pulmonary circulation are being metabolised locally in these patients. Indeed, in experimental cirrhosis, there is evidence of increased expression of endothelin type B receptors in the pulmonary circulation, and these are responsible for clearance of ET-1.² 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 vascular bed, 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^{3–4} as well as in the liver.⁵ 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.⁶ In addition, IL-6 promotes the coagulation cascade without affecting fibrinolysis, causing fibrin thrombi.⁶ 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.

F Wong

Correspondence to: Dr F Wong, University of Toronto, 9EN/220, Toronto Hospital, 200 Elizabeth St, Toronto, Ontario, Canada; florence.wong@utoronto.ca

Conflict of interest: None declared.

References

- 1 **Vaughan RB, Angus PW, Chin-Dusting JPF.** Evidence for altered vascular responses to exogenous endothelin-1 in patients with advanced cirrhosis with restoration of the normal vasoconstrictor response following successful liver transplantation. *Gut* 2003;**52**:1505–10.
- 2 **Luo B, Liu L, Tang L, et al.** Increased pulmonary vascular endothelin B receptor expression and responsiveness to endothelin-1 in cirrhotic and portal hypertensive rats: a potential mechanism in experimental hepatopulmonary syndrome. *J Hepatol* 2003;**38**:556–63.
- 3 **Martinet JP, Legault I, Cernacek P, et al.** Changes in plasma endothelin-1 and big endothelin 1 induced by transjugular intrahepatic portosystemic shunts in patients with cirrhosis and refractory ascites. *J Hepatol* 1996;**25**:700–6.
- 4 **Nagasue N, Dahr DK, Yamanoi A, et al.** Production and release of endothelin-1 from the

gut and spleen in portal hypertension due to cirrhosis. *Hepatology* 2000;**31**:1107–14.

- 5 **Gerbes A, Moller S, Gulberg V, et al.** Endothelin-1 and 3 plasma concentrations in patients with cirrhosis: role of splanchnic and renal passage and liver function. *Hepatology* 1995;**21**:735–9.
- 6 **Kerr R, Stirling D, Ludlam CA.** Interleukin 6 and haemostasis. *Br J Haematol* 2001;**115**:3–12.
- 7 **Streetz KL, Tacke F, Leifeld L, et al.** Interleukin 6/gp130-dependent pathways are protective during chronic liver diseases. *Hepatology* 2003;**38**:218–29.

Oesophageal entrapment of wireless capsule endoscopy in valvular patients

Wireless capsule endoscopy is an emerging new method of examining the small bowel. Its indications are currently widening, and occult and gastrointestinal bleeding of obscure origin, chronic diarrhoea and malabsorption syndromes, and suspicion of a small bowel neoplasm are now accepted indications.¹ Complications are rare, the main one being that it can become stuck in strictures or diverticulae inaccessible to flexible endoscopic retrieval.² The incidence in published series range from 1% to 5%, and all were managed with a surgical or endoscopic procedure.^{2–3}

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 for a capsule 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 capsule 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

Figure 1 The capsule was retained in the second third of the oesophagus for approximately four hours (the scan was taken nearly two hours after the obstruction).

Figure 2 The capsule became stuck in the distal oesophageal, retained by an extrinsic stricture.

was also suboptimal because the patient had ingested some fluids and food that interfered with small bowel vision.

This complication of wireless capsule endoscopy has not previously been reported. An elongated aorta was probably the cause of extrinsic pulsate compression of the thoracic oesophagus in our first case, and a dilated auricular cavity in the second. In both patients upper endoscopy had been performed twice and no strictures were identified.

Although not a life threatening complication, it limited the procedure results in both cases, leading to suboptimal results. A plain chest x ray or accurate oesophageal radiographic contrast studies, previous to the wireless capsule endoscopy procedure, could have provided clues. In such patients, it is advisable to assure passage into the stomach of the capsule on x ray. If the capsule is retained, water could be given to the patient to drink followed by a repeated plain chest x ray to exclude oesophageal entrapment of the capsule. Nevertheless, in a previous report, capsule impaction at the cricopharynx was described and the solution adopted by clinicians was endoscopic placement of the capsule in the stomach.⁴ This could be a feasible solution for patients in whom the capsule is stuck in an extrinsic vascular stenosis in the oesophageal lumen.

E Redondo-Cerezo, A Pérez-Sola, C Gómez, G Pérez-Vigara, J I Pérez-García, J Morillas, J A González-Martín

Digestive Diseases Department, Gastrointestinal Endoscopy Unit, Hospital General Virgen de la Luz, Cuenca, Spain

Correspondence to: Dr E Redondo-Cerezo, Sección de Aparato Digestivo, Unidad de Endoscopias, Hospital General Virgen de la Luz, C/ Hermandad de Donantes de Sangre, 16002-Cuenca, Spain; eredondoc@yahoo.es

doi: 10.1136/gut.2004.051318

Conflict of interest: None declared.

References

- 1 Gostout Ch J. Capsule endoscopy. *Gastrointest Endosc* 2002;10:2.
- 2 Swain P. Wireless capsule endoscopy. *Gut* 2003;52:iv48–50.
- 3 Pennazio M, Santucci R, Rondonotti E, et al. Outcome of patients with obscure gastrointestinal bleeding after capsule endoscopy: Report of 100 consecutive patients. *Gastroenterology* 2004;126:643–53.
- 4 Fleischer DE, Heigh RI, Nguyen CC, et al. Video-capsule impaction at the cricopharynx: a first report of this complication and its successful resolution. *Gastrointest Endosc* 2003;57:427–8.

Endogenous heparinoids in acute variceal bleeding

The risk of variceal bleeding in cirrhotics 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.^{3,4} 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.¹ 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 same sample 90 minutes after venepuncture⁵: a heparin effect was defined as an improvement in r time, k time, and α 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 “ α angle” (the slope drawn

Figure 1 Comparison of standard and heparinase I modified thromboelastography with respect to r time in two patients with variceal bleeding.

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 later); k time from 3.1 minutes to 10.7 minutes; and α angle from 50.8° to 17.8°. These values slowly returned to baseline concomitantly with disappearance of the heparin effect after 6–7 days.

Routine coagulation parameters (prothrombin time, activated partial thromboplastin time) did not show any correlation with worsening TEG parameters or the heparin effect.

The presence of endogenous heparinoids in cirrhotic patients with acute variceal bleeding is clearly demonstrated. There was no evidence of infection but the antibiotic prophylaxis possibly prevented or treated infection. Neither patient experienced early rebleeding. The heparin effect 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.³ 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 variceal bleeding or early rebleeding. It is possible that the heparin effect might be worse in the absence of antibiotics. This phenomenon deserves wider study, particularly as bacterial infection has been linked to failure to control variceal bleeding and early rebleeding, in a randomised study of prophylactic antibiotics.⁶

U Thalheimer, C Triantos, D Samonakis,
D Patch, A K Burroughs

Liver Transplant and Hepatobiliary Medicine Unit,
Royal Free Hospital, London, UK

A Riddell, D Perry

Haemophilia Unit, Royal Free Hospital, London, UK

Correspondence to: Professor A K Burroughs, Liver
Transplant and Hepatobiliary Medicine Unit, Royal
Free Hospital, Pond St, London NW3 2QG, UK;
Andrew.Burroughs@royalfree.nhs.uk

doi: 10.1136/gut.2004.051474

Conflict of interest: None declared.

References

- Bernard B, Grangé JD, Khac EN, *et al.* Antibiotic prophylaxis for the prevention of bacterial infections in cirrhotic patients with gastrointestinal bleeding: a meta-analysis. *Hepatology* 1999;29:1655–61.
- Gouliis J, Patch D, Burroughs AK. Bacterial infection in the pathogenesis of variceal bleeding. *Lancet* 1999;353:139–42.
- Montalto P, Vlachogiannakis J, Cox DJ, *et al.* Bacterial infection in cirrhosis impair coagulation by a heparin effect: a prospective study. *J Hepatol* 2002;37:463–70.
- Zambruni A, Thalheimer U, Coppell J, *et al.* Endogenous heparin activity detected by anti-Xa assay in infected cirrhotic and non-cirrhotic patients. *Hepatology* 2003;38:412A.
- Mancuso A, Fung K, Cox D, *et al.* Assessment of blood coagulation in severe liver disease using thromboelastography: use of citrate storage

versus native blood. *Blood Coagul Fibrinolysis* 2003;14:211–16.

- Hou MC, Lin HC, Liu TT, *et al.* Antibiotic prophylaxis after endoscopic therapy prevents rebleeding in acute variceal hemorrhage: a randomized trial. *Hepatology* 2004;39:746–53.

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 lymphoma.^{1–3} Later this condition was found to be associated with malabsorption as well as the presence of alpha heavy chain proteins.^{2–7} The disease was named by the WHO as “immunoproliferative small intestinal disease” (IPSID).⁶ One of the earliest reports of IPSID was from our centre.^{3,4} 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 one of 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.⁸

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%) colonic 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

Figure 1 Incidence rate of immunoproliferative small intestinal disease (IPSID) in the Fars province, Iran, from 1983 to 1999.

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. The 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 ($r^2 = 0.26$, $t(14) = -2.25$, $p = 0.04$).

IPSID was once the most common small intestinal malignancy in the Middle East.^{1–6} 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.⁷ 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 80% a decade later.⁹ Vaccination programmes increased dramatically after the Islamic revolution, reaching more than 90% of children.⁹ Local health facilities increased dramatically during the first two decades after the revolution.⁹

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

Age group (y)	Total	ASR	Males	ASR	Females	ASR
0–4	2	0.02	1	0.02	1	0.02
5–9	6	0.05	6	0.1	0	0
10–14	5	0.05	4	0.08	1	0.02
15–19	14	0.17	9	0.21	5	0.12
20–24	35	0.54	14	0.43	21	0.64
25–29	35	0.59	17	0.62	18	0.56
30–34	11	0.25	7	0.31	4	0.18
35–39	16	0.43	11	0.58	5	0.27
40–44	11	0.38	8	0.54	3	0.21
45–49	10	0.42	7	0.57	3	0.26
50–54	7	0.32	7	0.61	0	0
55–59	5	0.28	3	0.31	2	0.25
60–64	2	0.12	2	0.22	0	0
>65	2	0.13	2	0.25	0	0
Total	161	0.21	98	0.25	63	0.170

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

We postulate that improvement in health in general and decreasing childhood gastroenteritis 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.

**K B Lankarani, S M Masoompour,
M B Masoompour, R Malekzadeh, S Z Tabei,
M Haghsheenas**

Gastroenterology and Hepatology Research Center,
Shiraz University of Medical Sciences, Shiraz, Islamic
Republic of Iran

Correspondence to: Dr K B Lankarani,
Gastroenterology and Hepatology Research Center,
Shiraz University of Medical Sciences, PO Box 71345-
1414, Shiraz, Islamic Republic of Iran;
Lankarani@sums.ac.ir

doi: 10.1136/gut.2004.050526

Conflict of interest: None declared.

References

- 1 Azar HA. Cancer in Lebanon and the near east. *Cancer* 1962;15:66-78.
- 2 Seligmann M, Danon F, Huroz D, et al. Alpha chain disease: a new immunoglobulin abnormality. *Science* 1968;162:1396-7.
- 3 Haghighi P, Nabizadeh I, Asvadi S, et al. Cancer in Southern Iran. *Cancer* 1971;27:965-73.
- 4 Nasr K, Haghighi P, Bakhshandeh K, et al. Primary lymphoma of the upper small intestine. *Gut* 1970;11:673-8.
- 5 Haghsheenas M, Haghighi P, Abadi P, et al. Alpha heavy chain disease in Southern Iran. *Am J Dig Dis* 1977;22:866-73.
- 6 World Health Organization. Alpha heavy chain disease and related small intestinal lymphoma: a memorandum. *Bull WHO* 1976;54:615-24.
- 7 Khojasteh A, Haghsheenas M, Haghighi P. Immunoproliferative small intestinal disease, a third world lesion. *N Engl J Med* 1983;308:1401-5.
- 8 Boyle P, Parkin DM. Statistical methods for registries. In: Jensen OM, Parkin DM, eds. *Cancer registration principles and methods*. IARC Scientific Publications No 95. Lyon: IARC, 1991:126-58.
- 9 Marandi A, Azizi F, Larijani B, et al. *Health in the Islamic Republic of Iran*. Tehran: WHO/UNICEF, 1998.

Microscopic (collagenous and lymphocytic) colitis triggered by food allergy

Collagenous and lymphocytic colitis^{1,2} are rare³ diseases of unknown aetiology but several issues,^{1-3,5} in particular the good response to budesonide,⁷ are suggestive of immunopathology. Patients have watery

diarrhoea without abnormal findings on colonoscopy¹⁻⁵ but with increased numbers of intraepithelial lymphocytes, mast cells, and eosinophils⁵ 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 measured⁸ on a normal and hypoallergenic potato-rice diet. Colonoscopy with endoscopically guided segmental lavage for intestinal IgE was carried out⁹ and biopsies were investigated by routine pathology (haematoxylin-eosin), immunohistochemistry for eosinophil peroxidase, and amount of eosinophil cationic protein and tryptase⁹ in the whole biopsy. Clinical activity was mainly assessed by number of stools/day⁷ 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.

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 is 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, eosinophilic 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.⁶ Allergens could induce increased eosinophil infiltration and

Table 2 Number of stools per day (Baerts score⁷) before and after therapy

Sex	Age (y)	Before diagnosis	After diagnosis
F	49	5-10	1-2
M	45	5	0
M	46	4-6	0-1
M	52	10	1
M	71	4	1
F	59	5	1

enhance transforming growth factor β^{10} with increased collagen deposition. Eosinophils are highly susceptible to steroids which may explain the good response of collagenous colitis to budesonide.⁴

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.

M Weidenhiller

Clinic for Internal Medicine A, Ernst-Moritz-Arndt-University, Greifswald, Germany, and Department of Medicine I, University of Erlangen-Nürnberg, Erlangen, Germany

S Müller

Institute for Pathology, University of Erlangen-Nürnberg, Erlangen, Germany

D Schwab, E G Hahn, M Raithe

Department of Medicine I, University of Erlangen-Nürnberg, Erlangen, Germany

S Winterkamp

Department of Medicine I, University of Erlangen-Nürnberg, Erlangen, Germany, and Fachkrankenhaus Kloster Grafschaft, Pneumology and Weaning Centre, Schmallenberg, Germany

Correspondence to: Dr M Weidenhiller, Clinic of Internal Medicine A, Friedrich-Loeffler-Str 23 A, DE-17489 Greifswald, Germany; michael.weidenhiller@uni-greifswald.de

doi: 10.1136/gut.2004.049767

Conflict of interest: declared (the declaration can be viewed on the Gut website at <http://www.gut.com/supplemental>).

Table 1 Patient characteristics

Sex	Age (y)	Follow up (y)	Atopy	Signs of allergy	Diagnostic markers suggestive of allergy	Identified allergen
F	49	5	+	Improvement by hypoallergenic diet	Eos in bx	Egg, pollen, housedust mite
M	45	5	—	Urticaria, improvement by cromolyn	UMH	—
M	46	5	—	Pansinusitis	Eos in bx	Spices, moulds, flours, celery, nuts, milk, maize, rice, apple, celery, soy
M	52	3	—	IgG deficiency	Eos in bx	Maize starch
M	71	9	+	Prick	Prick, RAST	Milk, egg, soy
F	59	4	+	Prick	UMH	Ananas

Eos in bx, eosinophils in colon biopsy; UMH, urine methylhistamine.

References

- 1 Lindström CG. "Collagenous colitis" with watery diarrhea—a new entity. *Pathol Eur* 1976;11:87–9.
- 2 Read NW, Krejs GJ, Read MG, et al. Chronic diarrhea of unknown origin. *Gastroenterology* 1980;78:264–71.
- 3 Fernandez-Banares F, Salas A, Forne M, et al. Incidence of collagenous and lymphocytic colitis: a 5-year population-based study. *Am J Gastroenterol* 1999;94:418–23.
- 4 Schwab D, Raiithel M, Hahn EG. Kollagene und lymphozytäre Kolitis (Collagenous and lymphocytic colitis). *Deut Med Wochenschrift* 1998;123:1285–91.
- 5 Schiller LR. Pathophysiology and treatment of microscopic-colitis syndrome. *Lancet* 2000;355:1198–9.
- 6 Bonderup OK, Folkersen BH, Gjersøe P, et al. Collagenous colitis: a long-term follow-up study. *Eur J Gastroenterol Hepatol* 1999;11:493–5.
- 7 Baert F, Wouters K, D'Haens G, et al. Budesonide in collagenous colitis: a double-blind placebo-controlled trial with histologic follow-up. *Gastroenterology* 2002;122:20–5.
- 8 Weidenhiller M, Stolper J, Winterkamp S, et al. Urine-methylhistamine as an ambulant, non-invasive marker for food allergy. *Gut* 2001;49(suppl III):2764.
- 9 Schwab D, Raiithel M, Klein P, et al. Immunoglobulin E and eosinophilic cationic protein in segmental lavage fluid of the small and large bowel identify patients with food allergy. *Am J Gastroenterol* 2001;96:508–14.
- 10 Sampson HA. Food allergy. Part 1: Immunopathogenesis and clinical disorders. *J Allergy Clin Immunol* 1999;103:717–28.

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 colonoscopy 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).

Figure 1 (A) Magnifying chromoendoscopy using the contrast method. Indigo carmine spray reveals a neoplastic pattern. However, the pit pattern cannot be observed in some parts of the tumour. (B) After acetic acid spray, the detailed structure of the entire surface is immediately demonstrated more meticulously than with the contrast method. (C) Polyp covered with mucus. Dye spray using indigo carmine fails to reveal the surface structure because of the mucus. (D) After acetic acid spray, the mucous has been clearly removed and the surface of the polyp can be evaluated.

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!"

Y J Kawamura, K Togashi, J Sasaki, F Konishi
Jichi Medical School, Saitama, Japan

Correspondence to: Dr Y J Kawamura, Jichi Medical School, 1-847, Amanuma-cho, Omiya-ku, Saitama-shi, Saitama 3308503, Japan; kawamura@omiya.jichi.ac.jp

Conflict of interest: None declared.

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.¹ 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 photocoagulation of polyps. At that time, we were aware of a case of cap polyposis that was successfully treated with infliximab.² 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

Figure 1 Typical aspect of cap polyposis recurring in the rectum after sigmoid resection. Polyps are red, sessile, and covered with an exudate.

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 colleagues² 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.^{3,4} 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.⁵ It has therefore been suggested that abnormal colonic motility may be an important aetiological factor.

V Maunoury, M Breisse, P Desreumaux,
L Gambiez, J-F Colombel

Clinique des Maladies de l'Appareil Digestif et al de la Nutrition, Centre Hospitalier Universitaire Lille, Lille, France

Correspondence to: Professor J-F Colombel, Clinique des Maladies de l'Appareil Digestif et al de la Nutrition, Centre Hospitalier Universitaire Lille, CH et al U Lille, 59037, Lille, France; jfcolombel@chru-lille.fr

doi: 10.1136/gut.2004.053686

Conflict of interest: None declared.

References

- Geheot M, Colombel JF, Wolschies E, *et al*. Cap polyposis occurring in the postoperative course of pelvic surgery. *Gut* 1994;**35**:1670–2.
- Bookman I, Redston M, Greenberg GR. Successful treatment of cap-polyposis with infliximab: a case report. *Gastroenterology* 2004;**126**:1868–71.
- Ohkawara T, Kato M, Nakagawa S, *et al*. Spontaneous resolution of cap-polyposis: case report. *Gastrointest Endosc* 2003;**57**:599–602.
- Sadamoto Y, Jimi S, Harada N, *et al*. Asymptomatic cap-polyposis from the sigmoid colon to the cecum. *Gastrointest Endosc* 2001;**54**:654–6.
- Oriuchi T, Kinouchi Y, Kimura M, *et al*. Successful treatment of cap-polyposis by avoidance of intraluminal trauma: clues to pathogenesis. *Am J Gastroenterol* 2000;**95**:2095–8.

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".^{1–3} In their recent paper (*Gut* 2004;**53**:70–7), Di Sabatino *et al* showed that infliximab reverted defective peripheral and lamina propria lymphocyte apoptosis in CD patients via a caspase dependent mechanism, further corroborating the findings of previous studies.^{1–4} 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,⁴ provides a possible molecular explanation for the lack of efficacy of etanercept in a randomised placebo controlled trial in active CD.⁵

Figure 1 Intestinal CD4⁺ T cells were obtained from colonic biopsies from five patients with Crohn's disease (median age 34 years (range 18–49)) with an established diagnosis of Crohn's disease based on histopathological and endoscopic criteria. All patients had active disease at inclusion and were treated with 5-ASA (2–4 g/day, five patients), prednisone (20 mg/day, one patient), and azathioprine (150–200 mg/day, four patients). None of the patients had received anti-TNF- α treatment. Neither antigen nor feeder cells were added to the intestinal T cells cultures, which consisted of more than 97% CD3⁺/CD4⁺ T cells. (A) Infliximab, but not etanercept, activated p38 α and induced apoptosis in intestinal T lymphocytes from Crohn's disease patients. Levels of (phosphorylated) p38 mitogen activated protein kinase (MAPK) and PARP in *in situ* activated intestinal T lymphocytes were investigated by western blot 24 hours after stimulation with the respective TNF- α binding agent, as described previously.³ Data are representative of experiments performed in all patients, n = 5. (B) Signal transducer and activator of transcription 3 (STAT3) was activated in CD4⁺ cells in the intestinal mucosa in Crohn's disease patients *in vivo* and downregulated by both infliximab and etanercept in intestinal T cells from Crohn's disease patients *in vitro*. Western blot and immunofluorescence staining were performed as described previously.³ Filled arrowheads, cells positive for phospho-Y-STAT3 (anti-phospho-Y-STAT3, 1/100; Cell Signaling Technology) and CD4 (anti-CD4, 1/500; BD PharMingen, San Diego, California, USA); open arrowhead, CD4 immunoreactivity only (n = 5 for immunofluorescence and western blot; representative result for all experiments).

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 vivo³). Responders and non-responders to infliximab differ in the pattern of mucosal p38MAPK target phosphorylation, but not caspase-3 activation, further emphasising the complex modulation of intracellular signalling pathways beyond mere neutralisation of sTNF- α .⁶ 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.^{7,8}

According to the findings of van den Brande *et al.*,⁴ we observed PARP cleavage as a molecular hallmark of apoptosis in cultures grown with infliximab (fig1A) 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 p38MAPK 24 hours after infliximab treatment was observed in 4/5 of the cell lines derived from CD patients (fig1A).

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.⁸ 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 might be 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 inflammation.⁸

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 complex 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.

P Rosenstiel

Institute of Clinical Molecular Biology, University Hospital Schleswig-Holstein, Campus Kiel, Kiel, Germany

J Agnholt, J Kelsen

Department of Medicine V, Aarhus University Hospital, Aarhus, Denmark

V Medici

Institute of Clinical Molecular Biology, University Hospital Schleswig-Holstein, Campus Kiel, Kiel, Germany

G H Waetzig, D Seegert

Conaris Research Institute, Kiel, Germany

S Schreiber

Institute of Clinical Molecular Biology, University Hospital Schleswig-Holstein, Campus Kiel, Kiel, Germany

Correspondence to: Professor S Schreiber, Institute of Clinical Molecular Biology, University Hospital Schleswig-Holstein, Campus Kiel, Schittenhelmstrasse 12, 24105 Kiel, Germany; s.schreiber@mucosa.de

Conflict of interest: None declared.

References

- 1 ten Hove T, van Montfrans C, Peppelenbosch MP, *et al.* Infliximab treatment induces apoptosis of lamina propria T lymphocytes in Crohn's disease. *Gut* 2002;**50**:206–11.
- 2 Luger A, Schmidt M, Luger N, *et al.* Infliximab induces apoptosis in monocytes from patients with chronic active Crohn's disease by using a caspase-dependent pathway. *Gastroenterology* 2001;**121**:1145–57.
- 3 Waetzig GH, Seegert D, Rosenstiel P, *et al.* p38 mitogen-activated protein kinase is activated and linked to TNF- α signaling in inflammatory bowel disease. *J Immunol* 2002;**168**:5342–51.
- 4 Van den Brande JM, Braat H, van den Brink GR, *et al.* Infliximab but not etanercept induces apoptosis in lamina propria T-lymphocytes from patients with Crohn's disease. *Gastroenterology* 2003;**124**:1774–85.
- 5 Sandborn WJ, Hanauer SB, Katz S, *et al.* Etanercept for active Crohn's disease: a randomized, double-blind, placebo-controlled trial. *Gastroenterology* 2001;**121**:1088–94.
- 6 Waetzig GH, Rosenstiel P, Nikolaus S, *et al.* Differential p38 mitogen-activated protein kinase target phosphorylation in responders and nonresponders to infliximab. *Gastroenterology* 2003;**125**:633–4.
- 7 Agnholt J, Dahlerup JF, Kallot K. The effect of etanercept and infliximab on the production of tumour necrosis factor alpha, interferon-gamma and GM-CSF in in vivo activated intestinal T lymphocyte cultures. *Cytokine* 2003;**23**:76–85.
- 8 Lovato P, Brender C, Agnholt J, *et al.* Constitutive STAT3 activation in intestinal T cells from patients with Crohn's disease. *J Biol Chem* 2003;**278**:16777–81.

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 active 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,¹ the observation that infliximab induces activation of a proinflammatory MAPK does not tally with the evidence that the guanilhydrazide c-Jun N terminal kinase/p38 inhibitor CNI-1493 induces clinical improvement in steroid resistant Crohn's disease patients.² The reason for this discrepancy probably lies in the complex and dichotomous 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,^{3,4} and it shares differential actions in different T cell subpopulations.⁴ 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 10 production, thus tilting the balance between Th1 and Th2 cytokines to the former.⁴

Since several lines of evidence have also suggested the proapoptotic role of the p38 pathway,⁵ Waetzig and colleagues⁴ hypothesised 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 differences in signal transduction downstream of p38MAPK are not related to infliximab induced immune cell apoptosis.⁴

Disturbances in STAT signalling pathways, which transduce the immunomodulatory messages of most of cytokines/cytokine receptors, have been shown to be involved in the pathogenesis of Crohn's disease. In particular, STAT3 and STAT4 proteins are constitutively activated in lamina propria CD4+ T cells from active Crohn's disease patients.⁶ The finding of Rosenstiel *et al.* that both infliximab and etanercept are able to reduce in vitro STAT3 tyrosine phosphorylation of 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 potential of infliximab and etanercept in downregulating STAT3 signalling is consistent with the data of van den Brande and colleagues¹ 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 intracellular 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

of inflammatory bowel disease is critical in the identification of selective targets and development of new and rational therapies for the treatment of Crohn's disease.

**A Di Sabatino, R Ciccocioppo, R Morera,
G R Corazza**

Gastroenterology Unit, IRCCS Policlinico S. Matteo,
University of Pavia, Pavia, Italy

Correspondence to: Dr A Di Sabatino,
Gastroenterology Unit, IRCCS Policlinico S Matteo,
University of Pavia, Pavia, Italy;
a.disabatino@smatteo.pv.it

Conflict of interest: None declared.

References

- 1 **van den Brande JMH**, Braat H, van den Brink GR, *et al*. Infliximab but not etanercept induces apoptosis in lamina propria T-lymphocytes from patients with Crohn's disease. *Gastroenterology* 2003;**124**:1774–85.
- 2 **Hommes D**, van den Blink B, Plasse T, *et al*. Inhibition of stress-activated MAP kinases induces clinical improvement in moderate to severe Crohn's disease. *Gastroenterology* 2002;**122**:7–14.
- 3 **ten Hove T**, van den Blink B, Pronk I, *et al*. Dichotomous role of inhibition of p38 MAPK with SB 203580 in experimental colitis. *Gut* 2002;**50**:507–12.
- 4 **Waezig GH**, Rosenstiel P, Nikolaus S, *et al*. Differential p38 mitogen-activated protein kinase target phosphorylation in responders and nonresponders to infliximab. *Gastroenterology* 2003;**125**:633–4.
- 5 **Wada T**, Penninger JM. Mitogen-activated protein kinases in apoptosis regulation. *Oncogene* 2004;**23**:2838–49.
- 6 **Lovato P**, Brender C, Agnholt J, *et al*. Constitutive STAT3 activation in intestinal T cells from patients with Crohn's disease. *J Biol Chem* 2003;**278**:16777–81.

BOOK REVIEW

Small and Large Intestine

Edited by G Lichtenstein, G Wu. Pennsylvania: Mosby, 2003, £62.99, pp 364. ISBN 0323018955

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 entrenchment 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 professor 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 subject matter 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.

S J Middleton

CORRECTION

doi: 10.1136/gut.2003.035113corr1

The spelling of one of the authors in the paper by Byrne *et al* (CD4⁺CD45RB^{Hi} 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 Manuokian should read R Manoukian.