

(right- vs left-sided colon) to the other (primary vs metastatic tumour). We have shown that the expression of leptin (also considered as a cytokine-like) receptor in colon tumour cells influences chemokine expression depending on the colon location and MMR status.^{10–11} Simultaneous increases in FoxP3, IL17, IL6 and TGFβ expression in the majority of sporadic CRC (microsatellite stable (MSS) and left-sided), argue for a disturbed immune response of CRCs and may suggest that IL17 overproduction in these tumours probably leads to exacerbation of the disease. Relative concentrations of IL6 and TGFβ in tissues balance the differentiation of coexisting CD4⁺RORγt⁺ Th-17 cells and regulatory CD4⁺FoxP3⁺RORγt⁺ IL17-negative T cells, the ratio of which was proposed to play a role in the regulation of immunoreactivity.¹² Taking all these data together, the immune response in CRCs with MSI-H should be considered different from that observed in MMR-proficient tumours. Unfortunately, MMR status (routinely assessed by using immunohistochemistry and/or PCR) has not been characterised by Chaput *et al*,⁸ and the level of IL17 in situ expression is not given in this study. Thus, the increase in TGFβ1 and IL6 in their series cannot be correlated with the level of IL17 nor with the MMR status. Whether the immune response in tissue is the same regardless of the colon tumour site^{10–11} or metastatic site deserves further investigation.

The mode of antigen presentation may contribute to the induction of tumour-associated antigen (TAA)-specific T cells with impact on T effector cells. A positive correlation between the number of antigen-presenting cells (APCs) and Foxp3⁺ T cells and an inverse relationship with the

systemic TAA T cell response has been reported.⁴ Therefore, it would also be of great interest to investigate the tumour-infiltrating myloid-derived suppressor cells with special reference to MMR status.^{1–3}

THE PLACE OF BLOOD IMMUNE MARKERS IN COLON CANCER

Studies on peripheral blood cells are still preliminary, and pTNM staging still remains the best prognostic marker for treatment decision in the management of CRCs. It is not clear whether T4reg cells from blood might influence in situ differentiation of T cells,⁸ or higher CD8⁺CD25⁺Foxp3⁺ T cells in CRC patients' blood might come from tumour site(s). Decreased cytotoxicity of peripheral blood cells is shown to result from downregulated expression of cytotoxic molecules (perforin, granzymeB and FasL) in patients with cancer,¹³ suggesting that immune cell subsets from blood and analysis of their function may provide useful tools to analyse the global anti-tumour immune response.

In the future, patient cohorts should be stratified according to the tumour phenotype—that is, MMR status, tumour cell surface receptors for hormones and/or chemokines and cytokines, and selected mutation (Kras) and/or methylation pattern of DNA. Doctors and basic researchers have to use human colon cancer as a study model, with novel monitoring and/or therapeutic strategies targeting Treg cells. Trials should be designed on peripheral blood immune cell screening because blood sampling is easier for both patients and doctors, although several difficulties have to be managed: how to standardise blood sampling in terms of the time

course of recovery, screening of cell subsets, and functional testing—that is, the universal or specific tumour antigen-mediated response.¹³

Competing interests: None.

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Role of hydrogen sulfide in visceral nociception

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Hydrogen sulfide (H₂S) is a malodorous and toxic gas. Nevertheless, recent evidence suggests that H₂S is an endogenous

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mediator, joining nitric oxide and carbon monoxide as a third gaseous transmitter, and is increasingly being recognised as a key regulator of several cell processes and organ functions under both normal and pathological conditions.

H₂S can be generated in mammalian tissues by a number of different processes. It can be enzymatically synthesised from cysteine by cystathionine γ-lyase (CSE),

cystathionine β-synthase (CBS) and 3-mercaptopyruvate sulfurtransferase in combination with cysteine aminotransferase. There are also inorganic sources of H₂S such as the non-enzymatic haem-dependent reduction of sulfur. Also from a gut perspective, H₂S can be generated by the colonic microbiota before diffusing from the lumen into the intestinal mucosa to reach sub-epithelial targets within the gut wall. However, counteracting the build up of endogenous H₂S and potential toxic actions are protective mechanisms that degrade H₂S, and this is especially true of the intestinal epithelium which is extremely efficient at reducing H₂S to thiosulfate.¹

Since the first description of a physiological function for H₂S in enhancing

N-methyl-D-aspartate (NMDA) receptor-mediated responses and facilitating the induction of long-term potentiation in the hippocampus,² a host of effects have been reported in almost every cell or organ system studied to date.³⁻⁵ Inhibitors of CSE and CBS have been used to demonstrate a role for endogenous H₂S, and its role as a physiological regulator of blood pressure is apparent in the CSE knockout mouse which develops hypertension.⁶ In the gastrointestinal (GI) tract H₂S-synthesising enzymes have been shown to be expressed in the enteric nervous system,⁷ and H₂S is released from gut tissue.⁸ It has anti-inflammatory,⁵ spasmolytic⁹ and prosecretory actions.⁷ The latter, because they were attenuated by capsaicin desensitisation or transient receptor potential vanilloid receptor 1 (TRPV1) receptor blockade, indicates that the effect of H₂S may be mediated by an action on extrinsic afferents expressing TRPV1.⁷ However, the inhibition of intestinal motility by H₂S was similar in wild-type and TRPV1 knockout mice, suggesting there may be multiple targets for H₂S in the GI tract.¹⁰

The study by Matsunami *et al* (see page 751) suggests that H₂S exerts pronociceptive action in the mouse gut.¹¹ This conclusion is based on the finding that intracolonic application of the H₂S donor NaHS increased visceral nociceptive behaviour that was accompanied by referred abdominal hyperalgesia and allodynia, responses linked to activation or sensitisation of T-type Ca²⁺ channels since they were blocked by mibefradil. Surprisingly visceral sensitivity was not affected when NaHS was delivered intraperitoneally rather than into the colon, despite similar effects when capsaicin was administered by either route. Only the responses to intracolonic NaHS were dependent on T-type Ca²⁺ channels despite evidence that the two mechanisms converge at some point since intraperitoneal NaHS enhanced the nociceptive responses evoked by intracolonic capsaicin application, a facilitation that again involved T-type Ca²⁺ channels. These observations raise a number of questions relating to the mechanism underlying the effect of H₂S on colonic afferent sensitivity, which will be discussed below.

PRONOCICEPTION VERSUS ANTINOCICEPTION

Previous studies have described an antinociceptive effect of H₂S mediated by the opening of K_{ATP} channels. Two studies by Distrutti *et al* document H₂S evoking a reduction in visceromotor responses to colonic distension in both normal rats and

hyperalgesic rats following resolution of trinitrobenzene sulfonic acid (TNBS)-induced colitis.^{12, 13} In both cases, intraperitoneal application of either NaHS¹² or the H₂S-releasing derivative of mesalamine, ATB-429,¹³ attenuated pain-related behaviour. This antinociceptive effect was reversed by both glibenclamide (a K_{ATP} channel antagonist) and L-NAME (N^G-nitro-L-arginine methyl ester) to block nitric oxide (NO) production,¹² an observation suggesting a role for NO in H₂S production and/or in visceral afferent sensitivity. However, a confounding factor is the effects that H₂S and NO have on colonic tone, both causing muscle relaxation and increased compliance. The latter could impact on the nociceptive responses to distension since wall tension would be lower in a more compliant gut. However, since the effect on pain behaviour occurred at lower concentrations of H₂S than the effect on intraluminal pressure, this, argue the authors, would indicate that the two effects are unrelated.¹² On the other hand, it is clear that the biomechanical properties of the tissue surrounding the sensory endings plays a major role in shaping mechanosensitivity and, since K_{ATP} channels play a major role in regulating both vascular and visceral smooth muscle, it remains possible that the antinociceptive actions of H₂S are influenced by smooth muscle behaviour. Only direct recordings from sensory endings will enable the complex interplay between neuronal excitability, biomechanical properties and H₂S to be resolved.

Why then the discrepancy between the study of Matsunami *et al*¹¹ and these earlier studies?^{12, 13} One possibility is in the methodology used to evoke nociceptive responses and differences in nociceptive intensity and modality. Distrutti *et al* used colonic distension to evoke visceromotor responses and saw no effect of intraperitoneal NaHS on spontaneous pain behaviour.¹² In contrast, Matsunami *et al* quantified pain-related behaviour in the absence of distension and also saw no effect of intraperitoneal NaHS.¹¹ Only following intracolonic NaHS was there an increase in spontaneous pain-related behaviour. In the absence of distension, it is unlikely that colonic tone would influence sensitivity to the same extent as that evoked by distension. Thus, any potential antinociceptive effects arising in response to colonic distension may have been obscured by a pronociceptive effect through some alternative mechanisms, very probably involving activation of chemosensitive afferents upon intracolonic NaHS administration.

Species differences may also play a part. NaHS relaxes rat aorta via a K_{ATP}-sensitive mechanism, while the relaxation in mouse aorta is K_{ATP} independent.¹⁴ It is possible that species-dependent differences in subunit composition of K_{ATP} channels alter their sensitivity to H₂S. The subunit assembly of these channels determines their metabolic sensitivity and pharmacological profiles, and as such may shape the sensitivity to H₂S, although this has not been directly examined.

There may also have been differences in the inflammatory state of the bowel in the studies describing pronociceptive versus antinociceptive effects of H₂S. However, Distrutti *et al* observed similar antinociceptive effects in normal and postinflammatory states.^{12, 13} Nevertheless, a dual role for H₂S has been reported in inflammation-induced somatic hyperalgesia: an endogenous pronociceptive action due to neutrophil invasion and a direct antinociceptive action due to K_{ATP}-dependent blockade of nociceptor sensitisation.¹⁵

DIRECT VERSUS INDIRECT EFFECTS

The pronociceptive effect after intracolonic application of H₂S could potentially have been caused by a direct effect on nociceptive endings in the gut wall. This would fit the earlier observation that the secretory response to H₂S depends upon TRPV1.⁷ However, for this to happen, the luminal H₂S would need to overcome the epithelial mechanisms that degrade and detoxify luminal H₂S. This might occur more readily in circumstances of increased epithelial permeability such as occurs in inflammation, and indeed Matsunami *et al* observed a trend to increased tissue accumulation of Evans blue in their study.¹¹ Alternatively, H₂S may not act directly on the afferent endings but evoke release of proalgesic mediators from the mucosal epithelium or subepithelial immune cells, which in turn triggers an increase in afferent excitability. Indeed, this was the conclusion of a preliminary electrophysiological study in which an increased intestinal afferents firing in response to NaHS was linked to release of ATP.¹⁶ Therefore, the cellular target for the pronociceptive effect needs to be determined in order to distinguish between direct effects at the level of the sensory nerve ending and effects secondary to release of mediators that in turn increase afferent excitability. In this respect, H₂S is readily membrane permeable and is likely to penetrate colonic epithelial cells and diffuse deeper to affect numerous cell types in the gut wall, influencing local blood flow, immune

function as well as neural reflex activity. This might explain the effect that H₂S has on leucocyte and endothelial molecules that underlie the anti-inflammatory effect of H₂S.¹⁷ However, just as with nociception, there are two sides of the inflammatory story, and endogenous H₂S can also be proinflammatory. Increased H₂S concentrations are associated with ulcerative colitis and colorectal cancer.¹⁸ Sulfate-reducing bacteria are ubiquitously present in gut and in some studies linked to inflammatory bowel disease.¹⁹

MOLECULAR TARGETS FOR H₂S

The pronociceptive and antinociceptive effects of H₂S are suggested to depend upon different molecular targets, with T-type Ca²⁺ channels implicated in the pronociceptive effect and K_{ATP} in antinociception. This interpretation is based upon the use of pharmacological tools that interact with channels and receptors involved in H₂S effects, which in the case of T-type Ca²⁺ channels and K_{ATP} channels are mibefradil and glibenclamide. However, this is very muddy water because these drugs lack selectivity and can have effects outside their presumed molecular target. Mibefradil, for example, has actions on potassium channels at concentrations below those used to block T-type Ca²⁺ channels.²⁰ In addition, the cardioprotective effects of mibefradil have been linked to K_{ATP} channel opening.²¹ As in a previous study from the same group,²² Matsunami *et al* attempted to rule out non-specific effects by examining the effect of NaHS on the T-type current in isolated sensory neurons.¹¹ They showed using patch-clamp electrophysiology that NaHS facilitated the T-current in dorsal root ganglia (DRGs), an effect blocked by 1 μM mibefradil. However, the concentration of H₂S needed to evoke this current was high (1.5 mM) and absent at both lower and higher concentrations. This effect may therefore not reflect events occurring in the sensory nerve terminal, especially if the cellular target for H₂S is not the sensory ending itself. T-type Ca²⁺ channels have been shown to play an important role in pain transmission by altering the neuronal excitation threshold.²³ One mechanism underlying the action of H₂S on these channels relates to their sensitivity to redox modulation. While reducing agents such as L-cysteine (which is also a substrate for H₂S-synthesising enzymes) sensitise T-type Ca²⁺ channels on peripheral nociceptors, oxidising agents alleviate pain by diminishing T-type currents.²³ Redox modulation also occurs at a variety of

other channels and receptors that are involved in transmission of visceral sensitivity, including K_{ATP} channels,²⁴ transient receptor potential (TRP) channels such as TRPC,²⁵ TRPV1 and ²⁶ TRPA1,²⁷ glutamate receptors²⁸ and acid-sensing ion channels (ASICs).^{29–30} H₂S is a strong reducing agent and its protective effect on the liver is associated with an improved balance between reduced and oxidised glutathione and increased expression of the redox protein thioredoxin.³¹ Therefore, some of its effects may be related to protection of thiol groups from oxidation and modification of cysteine residues. The primary target, if there is one, of H₂S has yet to be discovered. Potentially, it may be changes in intracellular metabolism which, dependent on the cell type, species and/or experimental conditions, may link to a variety of intracellular signalling cascades. This could explain the enormous variety of receptors, channels, transporters and release mechanisms which are reportedly modulated by a simple molecule such as H₂S.

FOR THE FUTURE

The anti-inflammatory and antinociceptive effects of H₂S provide the basis for the use of H₂S donors as therapeutic agents. A pronociceptive action of H₂S would certainly be a serious adverse effect of such treatments. On the other hand, blockade of H₂S release as a strategy to relieve visceral pain may result in loss of potential gut defence mechanisms. This is a dilemma that can only be resolved with adequate studies in experimental models. There is a need to perform studies on direct effects of H₂S on visceral afferents, channels and transporters that are expressed on visceral sensory neurons, functional studies on nociceptive behaviour after more site-directed application of H₂S and, last but not least, studies in humans. Only then will it be possible to determine whether an increase in H₂S by H₂S-releasing compounds or blockade of H₂S synthesis has any potential to be used as a therapeutic option to treat visceral hypersensitivity.

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Selective targeting of activated T cells in chronic intestinal inflammation

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Programmed cell death (apoptosis) has been implicated in normal biological processes as well as in the pathology of human diseases.¹ The characterisation of genes involved in apoptosis has been pursued intensively and led to the identification of two major classes of genes: the bcl-2 family and the caspase family. Caspases are proteases that cleave their target substrates at specific peptide sequences and during apoptosis the activation of caspases takes place in a cascade fashion, leading to nuclear engulfment and cell death. Thus, caspases represent key functional components of the apoptosis pathway in human cells.

Resistance against apoptosis is a key phenomenon in various chronic inflammatory diseases as well as cancer.^{2–4} In the immune system, it has been shown that T cell resistance against apoptosis may contribute for the perpetuation of inflammatory processes such as seen in patients with inflammatory bowel diseases.^{5–6} This resistance of T cells against programmed cell death is probably driven by proinflammatory cytokines such as interleukin 6 (IL6).⁷ However, the functional relevance of T cell resistance against apoptosis is highlighted by the finding that induction of T cell death is likely related to the therapeutic efficacy of anti-tumour necrosis factor (TNF) antibodies in patients with inflammatory bowel diseases.⁵

Based on the above data targeting of T cell death emerges as an attractive

approach for therapy and selective new molecules that induce T cell death appear highly warranted. In the present issue of *Gut*, Shteingart *et al*⁸ (*see page 790*) report on the successful therapeutic use of an IL2–caspase 3 fusion protein in experimental colitis. This molecule belongs to one of the several types of fusogenic proteins that have recently been developed for selective therapy in the field of cancer.^{9–10} These molecules have mostly similar mechanisms of action and can be classified into three groups: targeting peptides, transduction peptides and killing peptides. Generally, a combination of peptides from the targeting and killing groups can be designed. Most studies have created fusion peptides incorporating toxins from bacteria or plants, but more recently pro-apoptotic proteins, ie, Bax, Bak, Bik as well as caspase 3 have been tried. By activation of the effector protein caspase 3 various substrates are cleaved that subsequently lead to cell death (fig 1).

Shteingart *et al* showed that treatment with the IL2–caspase 3 chimeric protein ameliorated the disease activity index in experimental colitis induced by dextran sulfate sodium (DSS). Specifically, such treatment resulted in an improvement in the clinical signs of disease, which includes loss of body weight, bloody diarrhoea and colon shortening, in a dose-dependent fashion. Furthermore, the protein was active both in preventive and therapeutic settings in DSS-induced colitis. Reduction of colitis activity was associated with decreased neutrophil and macrophage infiltration to the inflamed tissue. In addition, a reduction in mRNA levels of the pro-inflammatory cytokines TNF and IL1 β was noted in the colon upon therapy. Finally, the ratio between pro- and anti-inflammatory

proteins (Bax/Bcl-2 ratio) was upregulated by the IL2–caspase 3 protein.

For targeting T cells the authors chose the high-affinity IL2 receptor, mostly expressed on activated lymphocytes. This approach used by Shteingart *et al* for treatment of experimental colitis addresses several key problems in immunotherapy simultaneously: selectivity, specificity and side effects. Specific apoptosis in T lymphocytes, which are pathogenic players in inflammatory bowel diseases, can be achieved by combination of IL2 with caspase 3. The fusion protein will bind to high-affinity IL2 receptors on activated T cells and induce death of these cells via caspase 3. Especially, inadequate T cell reactions to harmless antigens should be a main reason for chronic inflammatory processes in IBD and should be targeted by the IL2–caspase 3 chimeric protein. While lamina propria T cells in uninflamed gut, compared to peripheral blood cells, have an increased spontaneous apoptotic behaviour, due to passive apoptotic mechanism associated with IL2 withdraw, T cells in inflamed tissue are more resistant to apoptosis.^{5–7} The deletion of these long-lived T cells, which cause tissue destruction, should markedly reduce the inflammatory symptoms. However, this approach should not lead to complete depletion of all mucosal T cells and therefore not cause massive immunodeficiency with infections. In this context, in the Discussion section the authors describe that regulatory T cells are not depleted by treatment with the fusion protein, although these cells carry the IL2 receptor and are critically dependent on IL2 for their survival.^{11–12} The reason for this phenomenon is unclear, however.

Taken together, the authors have described an intriguing new concept to treat intestinal inflammation by targeting activated T cells via an IL2–caspase 3 chimeric protein. Although they provide no direct proof that T cell apoptosis occurs upon therapy, the demonstration of an increased bax/bcl-2 ratio suggests that T cell death explains the beneficial effects of the chimeric protein in colitis. Subsequent studies should address the

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