Eicosanoid profile of healing colon anastomosis and peritoneal macrophages in the rat

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Abstract
Because intraperitoneal administration of prostaglandin E_2 (PGE$_2$) has a negative influence on the healing of colonic anastomosis, the production of eicosanoid products in the healing rat colon after resection and anastomosis was studied using high performance liquid chromatography. Normal colonic tissue metabolises small amounts of arachidonic acid into cyclo-oxygenase and lipoxygenase products. After construction of an anastomosis, however, there is increased production of lipoxygenase products, while cyclo-oxygenase activity remains low. Increased amounts of PGE$_2$ and other cyclo-oxygenase products are not produced after anastomosis of the colon and probably do not play a major role in uncomplicated healing of the large intestine in the rat. During the first eight days of repair in the anastomosed colonic tissue, a statistically significant increase in 12-hydroxyeicosatetraenoic acid (12-HETE) production was found compared with control colon tissue (p=0·001). At the same time peritoneal macrophages from these rats showed increased 12-HETE production. Eicosanoid synthesis of peritoneal macrophages resembled eicosanoid synthesis of anastomosed colon taken from the same rat indicating that 12-HETE, in particular, may be of macrophage origin.

Leakage of colonic and rectal anastomoses is a major complication after large intestine surgery, but the reported incidences vary greatly. Using routine barium enema studies, leakage was detected in up to 50% of patients who underwent anterior resection of the sigmoid colon and rectum.[1] Clinically evident anastomotic leakage is associated with increased mortality and morbidity.[2] There are many factors that contribute to either healing or leakage of an anastomosis: blood supply, oxygen tension, type of sutures, bowel preparation, surgical technique, tension on the anastomosis, and patient condition.[2-4] All these factors influence the formation of connective tissue, the key product in wound healing.

Halsted[4] first showed the importance of the submucosal connective tissue in relation to anastomotic healing in 1887. The quantity and quality of collagen in the submucosal layer of the intestinal wall determines the strength of the intestine and its capacity to hold sutures.[5]

Many studies on collagen metabolism in intestinal anastomoses have been performed using rat models. For the first three days after intestinal anastomosis a reduction in the suture holding capacity was found.[6,7] During this early phase there was a significant decrease in the collagen concentration in the colon.[8,9] This reduction in collagen content is highest just proximal to the anastomosis and can also be detected further away. After the third day, a rapid increase in strength is observed. Collagen production is increased from the very first day after operation and is highest in the vicinity of the anastomosis.[10] Comparison of net amounts with the rate of synthesis indicates an increased breakdown of collagen.[10]

Local degradation of mature collagen may be the cause of the severing of sutures resulting in breakdown of the anastomosis.[8,11] When collagenase inhibitor was given a significant increase in the bursting pressure of a colonic anastomosis was found in rabbits.[12-14]

Factors that directly inluence the formation of collagenase are infection and inflammation. As expected high collagenase activity was found in the colonic mucosa in colitis and around infected anastomoses.[11,15] Inflammatory cells, for example macrophages, are known to produce collagenase after stimulation by bacteria.[16] This collagenase production is dependent on prostaglandin E_2 (PGE$_2$). Rats treated with PGE$_2$ showed a significantly weaker anastomosis of the colon on day three than control rats, but this finding was reversed by the addition of indomethacin, a prostaglandin synthesis inhibitor.[17]

To evaluate the possible role of prostaglandins in uncomplicated healing of colonic anastomosis, we decided to measure eicosanoid synthesis in colon tissue and peritoneal macrophages. Peritoneal macrophages were studied as a possible source of the eicosanoid products.

Material and methods
Male inbred Wag/Rij rats weighing 200–300 g were used. The animals had free access to a standard diet (Hope Farms, Woerden, The Netherlands) and to water before and after surgery. Anaesthesia was induced by ether inhalation. After median laparotomy, the colon was divided 2·5 cm above the peritoneal reflection and a standardised left colonic resection of 1 cm was carried out with end to end anastomosis. The end to end anastomosis was made with 12 interrupted inverting sutures through all layers, using 7×0 polypropylene (Prolene, Ethicon, West Germany). On days 1, 2, 4, 8, and 16 the animals were sacrificed to obtain peritoneal macrophages and colonic tissue. Peritoneal macrophages and colonic tissue from rats who had not undergone surgery were used as controls.

PREPARATION OF PERITONEAL MACROPHAGES
Peritoneal cells were collected during ether anaesthesia by lavage with 50 ml of cold phos-

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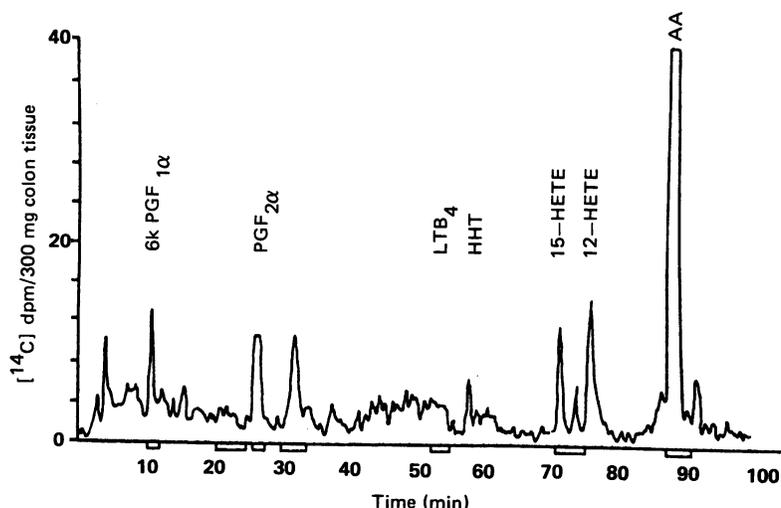
Figure 1: Reversed phase HPLC separation of eicosanoids produced by chopped normal rat colon tissue after loading with (^{14}C) arachidonic acid and Ca^{++}-ionophore A23187 challenge. Bars under time trace indicate peak width of both (^{14}C) labelled metabolites and (^{3}H) labelled standards.

Figure 3: Synthesis of prostanoids by chopped rat colon tissue after resection and anastomosis. Values are generated from reversed phase HPLC and given as percentage of the total region of interest (ROI). Each time point represents two tissue samples in one rat. Single measurements were performed.

phate buffered saline (PBS) (pH 7·2). The fluid containing the cells was centrifuged for 10 minutes at 250 g. The cells were resuspended in 5 ml cold PBS, washed once, and counted. The peritoneal macrophages were isolated from granulocytes by centrifugation and sedimentation on a Percoll gradient (Pharmacia, Sweden).[18]

PREPARATION OF COLON TISSUE

After peritoneal lavage, the abdomen was reopened and the colon was dissected free from the mesentery. Two segments of colonic tissue, each weighing 300 mg, were taken from the ascending and the descending colon. The latter, which contained the anastomosis, was removed, rinsed of blood with PBS, and immediately put on ice. The tissue was minced using a McIlwain tissue chopper and suspended in 5 ml PBS.
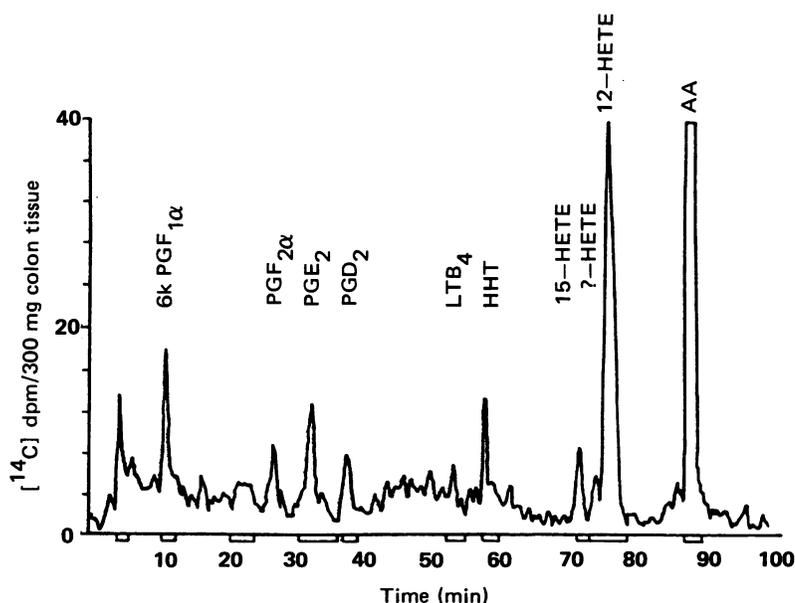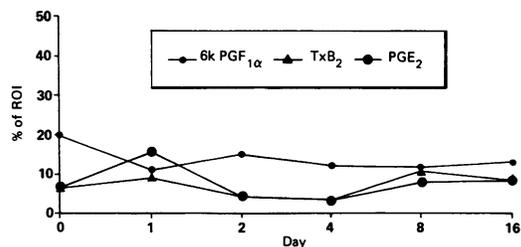
DETERMINATION OF EICOSANOIDS

Peritoneal macrophages and colonic tissue cells were incubated with 0·5 µCi (1-^{14}C) arachidonic acid and 2 µM calcium ionophore A23187 (Sigma, USA) in 10 ml Krebs Henseleit buffer at 37°C for 10 minutes. After this, known amounts of [3H]-labelled standards (thromboxane B_2, PGE_2, leukotriene B_4, and 12-hydroxy-5,8,10, 14,-eicosatetraenoic acid (12-HETE)) were added and the incubation sample was centrifuged for two minutes at 2800 g at 4°C. The supernatant was put in a SepPak C_{18} cartridge (Waters Associates, USA). The C_{18} cartridge was prewashed with 10 ml of absolute ethanol and 10 ml of water. The ethanol eluate was dried with a Savant Speed Vac concentrator, combined with a vacuum pump. The pellet was dissolved in 250 µl of methanol and filtered through a Gelman LC3a 0·45 µm filter (Gelman Sciences, USA) into a high performance liquid chromatography (HPLC) microvial. Of this sample, 100 µl was injected onto a Zorbax C_{18} HPLC column (250×4·6 mm id; Dupont, USA). Reversed phase chromatography was performed with a solvent system consisting of 30% acetonitrile in water, acidified with acetic acid to pH 2·4, and a flow of 1 ml/min at 37°C. After 35 minutes the acetonitrile was increased to 49% in 13 minutes and maintained for 40 minutes. Arachidonic acid was eluted at 100% acetonitrile. HPLC was performed using a Hewlett-Packard 1084B (USA) liquid chromatograph consisting of a double head pump, a temperature controlled column compartment, and a variable volume injector. An on-line Berthold LB 506c radioactivity monitor (Wildbad, West Germany) was controlled by the HP 1084B terminal.

Figure 2: Reversed phase HPLC separation of eicosanoids produced by chopped rat colon tissue one day after resection and anastomosis, in response to (^{14}C) arachidonic acid and Ca^{++}-ionophore A23187. Bars under time trace indicate peak width of both (^{14}C) labelled metabolites and (^{3}H) labelled standards.
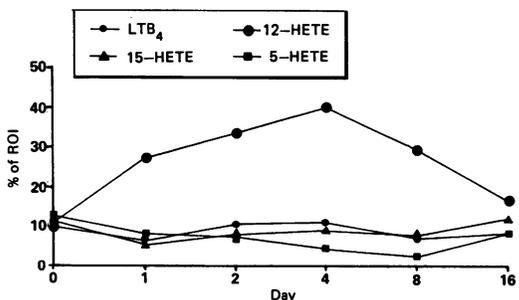
Figure 4: Synthesis of lipoxygenase products by chopped rat colon tissue after resection and anastomosis. Values are generated from reversed phase HPLC and given as percentage of the total region of interest (ROI). Each time point represents two tissue samples in one rat. Single measurements were performed.

Figure 5: Synthesis of lipoxygenase products by chopped rat colon tissue proximal to colon anastomosis after resection and anastomosis of the left colon. Values are generated from reversed phase HPLC and given as percentage of the total region of interest (ROI). Each time point represents two tissue samples in one rat. Single measurements were performed.
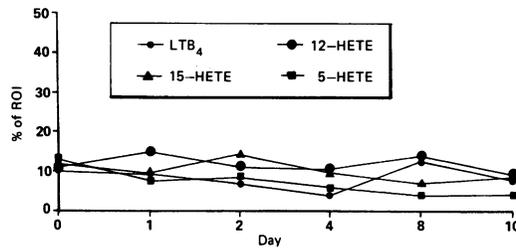

Figure 7: Synthesis of lipoxygenase products by rat peritoneal macrophages after resection and anastomosis of the left colon. Values are generated from reversed phase HPLC and given as percentage of the total region of interest (ROI). Each time point represents two tissue samples in one rat. Single measurements were performed.

STATISTICAL METHODS

Statistical analyses were performed using multi- and univariate analysis of variance (Anova). The null hypothesis of no difference is rejected at the value of 0·05. However, in the case of findings by chance, Bonferroni's inequality implies that the value of significance is $p = 0·0125$.[19]

Results

All animals survived the operation. The profile of eicosanoids synthesised by normal rat colon is shown in Figure 1. Normal rat colon tissue metabolises a small portion only of the exogenous arachidonic acid; approximately 15% is converted into a number of eicosanoids from which small peaks of 6 keto $PGF_{1\alpha}$, $PGF_{2\alpha}$, LTB_4, HHT (12-hydroxy-5,8,10-heptadecatrienoic acid), 15-HETE, 12-HETE, and 5-HETE can be detected. Of the lipoxygenase products, the 12-HETE is synthesised most prominently (40%). On day 1 after surgery the HPLC profile of the segment of colon containing the anastomosis had changed drastically: 6 keto $PGF_{1\alpha}$, PGE_2, PGD_2, and HHT tended to increase, but not significantly; 15-HETE had decreased; but there was a fourfold increase in production of 12-HETE (Fig 2).

In Figures 3 and 4 the values of the lipoxygenase products LTB_4, 15-HETE, 12-HETE,

and 5-HETE and prostanoids 6 keto $PGF_{1\alpha}$, TxB_2, and PGE_2 as calculated from the HPLC profiles are given as percentage of region of interest. The graph clearly shows the increased 12-HETE synthesis, reaching maximal differences from basic values on day 4 (statistically significant, $p < 0·001$). On day 16, 12-HETE synthesis in colonic anastomosis tissue had returned to normal values. In the segments taken proximal to the anastomosis only minor changes in production of eicosanoids occurred (Fig 5).

Eicosanoid synthesis by peritoneal macrophages shows a shift from cyclo-oxygenase to lipoxygenase products on day 1 compared with day 0. An example of an HPLC profile of eicosanoids synthesised by peritoneal macrophages is shown in Figure 6. Like colonic tissue at the site of the anastomosis, peritoneal macrophages from the same rat show a noticeable lipoxygenase activity, starting on day 1 (Fig 7). On day 8 the 12-HETE production is maximal. The other lipoxygenase products show minor variation.

Discussion

This study shows that normal rat colonic tissue metabolises a small portion only of exogenous arachidonic acid into cyclo-oxygenase and lipoxygenase products. The 6 keto $PGF_{1\alpha}$ was the major cyclo-oxygenase product but 12-HETE was the major lipoxygenase product detected. These findings agree with reports showing that the mucosa of normal rat intestine metabolises a very small portion only of exogenous arachidonic acid into cyclo-oxygenase products.[20] Although the proportion metabolised is still small, the profile of products changes after resection and anastomosis of the colon: our results show a sharp increase in 12-HETE production, whereas cyclo-oxygenase products show only minor increase.

PGE_2 administered intraperitoneally has been reported to have an adverse influence on the healing of colonic anastomosis in the rat.[17] Our results show, however, that PGE_2 and other cyclo-oxygenase products are not produced in excess after anastomosis of the colon and probably do not play a major role in uncomplicated healing of the large intestine in the rat.
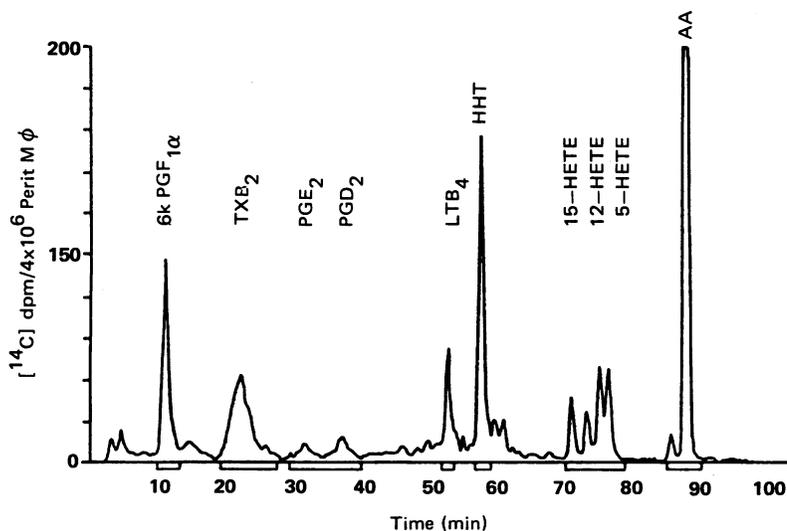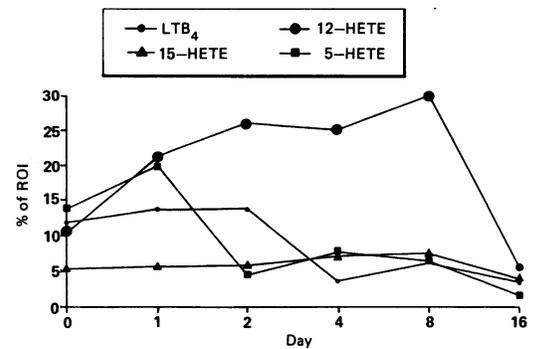

Figure 6: Reversed phase HPLC separation of eicosanoids produced by rat peritoneal macrophages obtained 1 day after colonic resection and anastomosis, in response to (^{14}C) arachidonic acid and Ca''-ionophore A23187. Bars under time trace indicate peak width of both (^{14}C) labelled metabolites and (^{3}H) labelled standards.

The origin and possible role of 12-HETE in wound healing are interesting issues. After tissue injury such as surgery, phospholipase A_2 is activated.[21] This phospholipase releases arachidonic acid from the phospholipid stores in cell membranes. The liberated arachidonic acid is the substrate of eicosanoids. The type of eicosanoids synthesised may vary greatly between different cell types.[22] During the first 24 hours after the construction of an anastomosis, the colonic wound is infiltrated by neutrophil granulocytes, after which eosinophils and macrophages gradually occur.[23] 5-Lipoxygenase products, LTC_4 and LTB_4, are produced in high amounts by all cell types mentioned, however, platelets synthesise mainly 12-HETE.[24] This means that basically any of these cell types may be responsible for the observed alteration of eicosanoid synthesis in our study.

The primary influx of granulocytes, which produce, in particular, the chemotactic leukotriene B_4, could be responsible for the LTB_4 formation. During the early phase of wound healing, however, we did not observe a significant increase in this product. Moreover, the pattern of the lipoxygenase arachidonic acid metabolites did not resemble that of stimulated peripheral blood neutrophils. It is possible that thrombocyte contamination of the sample tissue occurred, but in this case one would also expect high TxB_2 and HHT values and these were not noted in our study.

The results of this study show that normal colonic tissue does not itself have a significant lipoxygenase activity. Using a rat colitis model, LTB_4 and 5-HETE were found in the mucosa of the large intestine, whereas in normal rat colon mucosa no lipoxygenase products were found.[20] The enhanced intestinal prostanoid synthesis in inflammatory bowel disease is thought to come from stimulated local mononuclear cells.[25]

This leads us to our final candidate, the macrophage. Macrophages are a known source of lipoxygenase metabolites, 12-HETE being the major one produced. This strongly suggests that the increase in 12-HETE found in our study is of macrophage origin and that macrophages play an important role in colonic anastomosis healing from as early as day 1 after surgery. This is in contrast with the results of other investigators who found that in rat colonic anastomosis, monocytes are not seen for 48 hours.[26] To learn more about the presence of macrophages in the first few days after anastomosis more advanced studies, for example, using monoclonal antibodies against macrophages, are needed.

There is substantial evidence that some arachidonic acid metabolites cause or enhance the signs of inflammation, the first phase of wound healing.[22] Compared with other eicosanoids, there is little information on the function of 12-HETE. Although less active than LTB_4, 12-HETE also has chemotactic activity on inflammatory cells.[27] The products of the lipoxygenase pathway stimulate proliferation of epidermal cells and lymphocytes.[28] Topical administration of LTB_4 and 12-HETE stimulates epidermal proliferation.[29] In addition, it was found that monoHETEs, including 12-HETE, exert modulatory actions on arachidonic

acid metabolism in peritoneal macrophages.[30] Interestingly, 12-HETE inhibited synthesis of PGE_2. The mentioned properties of 12-HETE may indicate a possible role in wound healing. From this information, however, we cannot deduce whether 12-HETE plays a significant role in colon healing or whether it is a rather aspecific finding. As macrophages are a known source of collagenase, their activity in the healing colon may cause collagen breakdown in the early phase of wound repair.

It is evident that the raised lipoxygenase activity after the construction of a colonic anastomosis needs further study to determine whether other species and humans show the same phenomena. Such a study on the effect of lipoxygenase inhibition on intestinal wound healing is currently being undertaken.

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