Molecular mechanisms in colon cancer

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Chapter III

NF-kappaB, p38 MAPK and JNK are highly expressed and active in the stroma of human colonic adenomatous polyps

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Abstract

Background and aims: The factors that govern the progression from colonic adenomatous polyp to colon cancer are poorly understood. The observation that NSAIDs act as chemopreventative agents and reduce the size of colonic polyps suggests the involvement of inflammatory signalling, but inflammatory signalling in colonic polyps has not been studied.

Methods: We investigated the expression of the active forms of NF-κB, JNK and p38 MAPK using immunohistochemistry with activation specific antibodies in human colonic adenomas.

Results: We show that active NF-κB is seen in stromal macrophages that also express COX-2 and TNF-α, active JNK is seen in stromal and intraepithelial T-lymphocytes and periendothelial cells of new blood vessels, and active p38 MAPK is most highly expressed in macrophages and other stromal cells.

Conclusions: These results demonstrate the presence of active inflammatory signal transduction in colonic polyps and that these are predominantly in the stroma. In the case of NF-κB this coincides with the cellular localisation of COX-2. These results support evidence that NSAIDs may act through effects on stromal cells rather than epithelial cells.
Inflammation and signal transduction in colon polyps

Introduction

Non Steroidal Anti Inflammatory Drugs (NSAIDs) and other anti-inflammatory compounds such as Curcumin act as chemopreventative agents in colon cancer. This suggests a role for inflammation and inflammatory pathways in the progression of colon cancer. Surprisingly, the activity of inflammatory signal transduction pathways has not been studied in colon cancer. In this study we set out to study the site-specific activity of Nuclear Factor Kappa B (NF-κB), p38 Mitogen Activated Protein Kinase (p38 MAPK), and cJun NH₂ terminal kinase (JNK) in human colonic polyps. They are all components of signal transduction pathways that play pivotal roles in inflammation. That molecules within these pathways may also provide direct targets for NSAIDs and are important in regulating cell proliferation and apoptosis, makes the characterisation of their expression in colon cancer all the more interesting.

NSAIDs are thought to prevent colon cancer by their actions on Cyclooxygenase enzyme type 2 (COX-2). Mice treated with specific COX-2 inhibitors or bred to be genetically deficient in COX-2 develop dramatically fewer colon tumours than their littermates in a mouse model of familial adenomatous polyposis (APC mice) and over expression of COX-2 in intestinal epithelial cells leads to enhanced tumourigenic phenotypes, metastatic potential and angiogenesis. However, there is increasing evidence that NSAIDs have important COX independent effects both in their action as chemopreventative agents, and as anti-inflammatory agents. Several studies have shown specific inhibition of I kappa B kinase beta leading to inhibition of NF-κB thus interfering with the transcription of inflammatory cytokines. This finding, together with the knowledge that NF-κB plays an important role in apoptosis, has led many to list NF-κB as a possible COX independent mechanism for the action of NSAIDs in colon cancer. Others have found that Salicylates promote apoptosis via a p38 MAPK dependant mechanism and may also activate JNK.

The expression of JNK in colon cancer has already received some attention. The authors of these studies found increased activity of JNK in both rat models of colon cancer as well as human tumours. However their data comes from homogenates of tumour tissue as thus gives no indication as to where in the tumour these molecules are expressed and therefore less information as to how they might be important in the biology of colon cancer.

We performed immunohistochemistry using antibodies specific to the active forms of these proteins to identify which pathways are active in the polyps, and identify in which cells...
these pathways are active. This approach has only relatively recently become feasible with the advent of reliable ‘phospho-specific’ antibodies to signalling pathway components, and other antibodies that are highly specific to the active forms of these proteins.

We used immunohistochemistry as it has the advantage of giving an indication of which cell types within the polyps are expressing the active signalling components. This is particularly important in the light of the developing interest in the role of stromal tissue in colon cancer progression.\(^{131}\)

There is mounting evidence that the stroma, consisting of infiltrating inflammatory cells, new blood vessels and connective tissue cells, plays a vital role in the progression of colon cancer. COX-2 is found by many to be mainly expressed in the stromal tissue of polyps, predominantly in macrophages, both in the polyps of a mouse model of Familial Adenomatous Polyposis (Min mice),\(^{132}\) and also in human adenomatous polyps.\(^{133}\) \(^{134}\) In vivo evidence that the antitumour effects of NSAIDs may be brought about through actions on stromal cells rather than on the neoplastic cells comes from an elegant recent study showing that growth of a tumour xenograft was severely inhibited by disruption of host COX-2 production.\(^{135}\) In this model all the stromal cells are provided by the host, and a pure culture of tumour cells are injected as a xenograft. Thus it is of great importance to show in which cells molecules implicated in cancer progression are expressed, and not to rely solely on data from tissue homogenates.

**Materials and methods**

The ten most recent cases of adenomatous polyp removed at colonoscopy, together with normal biopsies from the same patients, where these existed, were taken from the archives of the Pathology Department at the AMC. 4 μm sections were prepared from the formalin fixed, paraffin embedded tissue and mounted on slides coated with polylysin. We took polyps rather than cancer specimens because evidence points towards NSAIDs exerting their effects during this relatively lengthy period in the progression of cancer. We took sporadic polyps by excluding specimens from patients with FAP and with Hereditary Non-Polyposis Coli (HNPPCC) as we reasoned that in order for NSAIDs to have the magnitude of effect that has been observed they must act on these polyps, which comprise well over 90% of cases.
Primary antibodies

a) Mouse monoclonal anti-human antibody to activated NF-κB (Boehringer-Mannheim, Mannheim, Germany). This antibody recognises an epitope overlapping the nuclear localisation signal of the p65 subunit of the NF-κB heterodimer. This epitope is masked by I kappaB and revealed when I kappaB is degraded. Thus the antibody selectively binds to the activated form of NF-κB. Concentration used 1:100.

b) Mouse monoclonal anti-human antibody to Phospho-JUN NH2 terminal kinase (Santa Cruz Biotechnology, Santa Cruz, CA). This antibody binds to the phosphorylated, active form of JNK and does not cross react with the unphosphorylated, inactive form. Concentration used 1:200.

c) Mouse monoclonal anti-human antibody to Phospho-p38 MAPK (Santa Cruz). This antibody is specific to the active, phosphorylated form of p38 MAPK. Concentration used 1:200.

d) Mouse monoclonal anti-human antibody to COX-2 (Cayman Chemical Co., Ann Arbor, MI). Concentration used 1:100.

e) Mouse monoclonal anti-human antibody to TNF-alpha clone 4C6-H6 (Instruchemie, Hilversum, The Netherlands). Concentration used 1:100.

f) Rabbit polyclonal anti-human antibody to CD3 (DAKO, Glostrup, Denmark). Concentration used 1:200.

g) Mouse monoclonal anti-human antibody to CD68 clone KP-1 (DAKO). Concentration used 1:200.

h) Mouse monoclonal anti-human antibody to CD31 clone JC/70A (DAKO). Concentration used 1:100.

Secondary antibodies

Goat anti-mouse antibodies and Goat anti-Rabbit antibodies conjugated with biotin or Alkaline Phosphatase depending on detection mechanism (All from DAKO).

Immunohistochemistry: Single stainings

Sections were dewaxed and rehydrated in graded alcohols. Endogenous peroxidase activity was quenched with 1.5% H2O2 in Phosphate Buffered Saline (PBS) for 30 mins and then washed in PBS. Non-specific binding sites were blocked with TENG-T (10mM Tris, 5 mM EDTA, 0.15 M NaCl, 0.25% gelatin, 0.05% (v/v) Tween 20, pH 8.0) for 30 mins, except
for the COX-2 stainings where a solution containing 10mM Tris, 0.1M MgCl2, 0.05% (v/v) Tween, 1% bovine serum albumin (BSA) and 5% (v/v) Goat serum, was used for one hour. Slides were then washed (3 x 5 mins in PBS). Slides were incubated at 4°C overnight with the primary antibodies in PBS with 0.1% Triton and 1% BSA. After washing (3 x 10 mins in PBS), slides were then incubated with the secondary antibody at room temperature for one hour in PBS with 10% Human serum. Slides were washed (3 x 5 mins in PBS), incubated with streptavidin-biotin-horseradish peroxidase (Dako) for 1 hour, washed again (3 x 5 mins in PBS), and peroxidase activity was detected with 3,3′-diaminobenzidine (Sigma, St. Louis, MO), 0.5mg/ml in 0.05M Tris Ph 7.6 with hydrogen peroxide (0.05%). Finally, sections were counterstained with Mayer’s hematoxylin, dehydrated and mounted in ‘Entellan’ (Merk, Darmstadt, Germany) under coverslips. Controls consisted of omitting the primary and secondary Ab and use of an appropriate Ig control.

**Immunohistochemistry: Double stainings**

NF-κB and pJNK double staining with CD68 and CD3 respectively, required the initial staining to be on the sections with no prior use of an antigen retrieval method. After the first staining the same sections were then digested with 0.25% Pepsin in 0.01M HCl at 37°C for 15 mins followed by washing (3 x 5 mins in PBS), before proceeding with the second staining. For the blue β-galactosidase staining biotinylated secondary antibodies were used and sections were then incubated with streptavidin β-galactosidase (1:40, Boehringer Mannheim) at room temperature for 30 min. Detection was with 1% X-gal (DAKO) in iron phosphate buffer (0.02% MgCl2·6H2O, 0.099% potassium ferricyanide, 0.127% potassium ferrocyanide) at 37°C for 15 min. For the ‘Fast Red’ detection method (DAKO) alkaline phosphatase conjugated secondary antibodies were used and after washing in Tris-buffered saline, the alkaline phosphatase was detected using the ‘Fast Red’ detection method. Double-stained sections were mounted in Ultramount (DAKO), an aqueous mounting medium.

**Counting of Lymphocyte numbers in all specimens**

Five digital pictures were taken at 200x magnification from random areas of slides prepared from each of the ten polyp specimens and from the six specimens of normal tissue stained with anti-CD3. These were scored blindly using image analysis software.
Results

**Active NF-κB is seen mainly in stromal macrophages**

Strong staining with anti-active NF-κB is seen in mainly in stromal cells within human colonic polyps. Figure 1 shows that these stromal cells are CD68 positive cells as seen in sequential sections (A and B) and double stained sections (C). These macrophages are situated both superficially in the lamina propria where they form aggregates in close proximity to capillaries, and also in the stroma deeper within the polyps where they are more widely and evenly spaced. The superficial aggregates are not seen in the normal tissue specimens, although normal specimens do have similar numbers of deep macrophages (D).

**Active NF-κB and COX-2 enzyme are expressed in the same cells**

COX-2 enzyme has been recently shown to be predominantly expressed in superficial macrophages in colonic polyps. We therefore decided to investigate whether there was any overlap between COX-2 and active NF-κB expression. Using staining in sequential sections we observed a very close pattern of expression of the two molecules. Figure 2 shows that active NF-κB (A and C) is seen in the nuclei of the same cells that express COX-2 (B and D), as judged from staining of sequential sections. Double staining methods were not used because the two antibodies are both mouse monoclonal antibodies of the same IgG subclass and thus difficult to distinguish with secondary antibodies.
Figure 1 Immunohistochemistry for active NF-kappaB in human sporadic colonic adenomas. Strong staining for active NF-κB is seen localised to aggregates of superficial stromal cells as well as stromal cells deeper within the polyps (a). These cells are macrophages as determined by morphology and by CD68 (a macrophage marker) staining in sequential sections (b) and lie in close proximity to capillaries. This was further confirmed by performing active NF-κB (blue) and CD68 (red) double staining (c). Normal colonic tissue (d) shows similar active NF-κB staining of deep macrophages but does not contain the superficial aggregates of macrophages. Throughout all immunohistochemistry control staining was consistently negative. An example performed by omitting the primary antibody (e) is shown.
Inflammatory signal transduction in colonic polyps

**Figure 2** Immunohistochemistry for active NF-κB (a+c) and COX-2 (b+d) in human sporadic colonic polyps. A remarkable degree of overlap was seen between the staining patterns of these two molecules in sequential sections. In the enlargements (c+d) of the areas indicated, cells appearing in both sections that stain positively for both molecules are indicated with arrows.

**Active NF-κB and TNF alpha are expressed in the same cells**

An established method to confirm that the active NF-κB antibody staining is specific is to look for expression of TNF alpha.\(^{136}\) TNF alpha both leads to NF-κB activation and is itself transcriptionally upregulated by active NF-κB. We show a close overlap of TNF alpha and NF-κB expression in sequential sections in Figure 3, and that both these molecules are predominantly active in CD68 positive macrophages.

**Phosphorylated JNK is seen mainly in stromal T-lymphocytes and periendothelial cells**

Staining with Phospho-JNK antibodies is shown in Figure 4. Strong staining is localised to the polyp stromal tissue (A). Morphologically this appeared to be largely in T-lymphocytes and intraepithelial T-lymphocytes. Staining of serial sections (C and D) showed a strong overlap between CD3 and pJNK staining and double staining (E and F) confirmed
that pJNK is predominantly found in stromal T-lymphocytes and intraepithelial T-lymphocytes. There was also strong staining of cells surrounding the microvasculature of the polyp. CD31 staining showed that these were cells lying outside endothelial cells, the periendothelial cells or pericytes (not shown). There were significantly larger numbers of T-lymphocytes infiltrating the colonic polyps compared to normal tissue (212; 95% CI 226-198 vs 48; 95% CI 36-60) as judged by counting CD3 positive cells. This difference largely accounts for the higher pJNK expression seen in polyps versus normal tissue (B)

**Figure 3** Immunohistochemistry for active NF-κB (a+d), TNF-α (b+e) and CD68 (c+f) in human sporadic colonic polyps. Sequential sections were stained for active NF-κB and TNF-α. These two molecules are expressed predominantly in the stroma and both in the same cells within the stroma. These cells are macrophages as shown by the macrophage marker (CD68) staining in a further sequential section (e). In the enlarged sections (d, e and f), white arrows mark cells that stain positively for both active NF-κB (d) and TNF-α (e), and black arrows mark one cell that appears across all three sections and another that stains positively for TNF-α (e) and the macrophage marker CD68 (f)
**Figure 4** Immunohistochemistry for phosphorylated JNK in human sporadic colonic polyps. Strong staining is seen localised to the stromal tissue (a), with no staining in the epithelial cells. Normal colonic tissue (b) shows lower levels of staining, with some stromal inflammatory cells staining positively. A close up image of pJNK staining shows strong staining of inflammatory cells infiltrating the polyp (c). Staining of sequential sections for the T-lymphocyte marker CD3 (d) confirms that these are T-lymphocytes. Cells that appear in both sections and stain positively for both CD3 and pJNK are indicated with arrows (e-d). Double staining for pJNK (blue) and CD3 (red) further confirms that pJNK is predominantly expressed in T-lymphocytes (e). An enlarged section of image e is shown in image f where pJNK and CD3 localise to the same cells.
**Phosphorylated p38 MAP kinase is most highly expressed in the stroma**

Phosphorylated p38 MAP kinase (pp38 MAPK) stains most strongly in the stroma. Here, as seen in Figure 5, staining of sequential sections with CD68 shows that some of these stromal cells are macrophages and that areas of stroma expressing high levels of pp38 MAPK are those where there are large numbers of CD68 positive macrophages. However, not all the pp38 MAPK positive cells are CD68 positive macrophages. Other stromal cell types also express pp38 MAPK, and there is also low-level expression in epithelial cells. Normal colonic epithelium shows a similar pattern of staining but the levels are lower and there are no superficial aggregates of macrophages and fewer inflammatory cells in general (C).

**Figure 5** Immunohistochemistry for phosphorylated p38 MAPK in human sporadic colonic polyps. Staining for pp38 MAPK is strongest in the stromal tissue with lower level staining in the epithelial cells (a). Many of these stromal cells are macrophages as shown by staining of sequential sections with the macrophage marker CD68 (b). Normal colonic tissue shows lower levels of staining, again predominantly in the stromal cells (c)
Discussion

The evidence that non-steroidal anti-inflammatory drugs (NSAIDs), such as Aspirin, prevent colon cancer\textsuperscript{31} provides a rational starting point for investigation into the molecular biology of colon cancer. These anti-inflammatory agents exert many of their effects through the inhibition of the enzymes COX-1 and 2 therefore research into their chemopreventative effects has also focussed heavily on these enzymes, resulting in powerful evidence that NSAIDs prevent colon cancer by inhibiting COX. However there are several findings that suggest that COX may not be the only answer. Sulindac Sulphone, with no appreciable COX inhibiting activity nevertheless retains its antitumour activity\textsuperscript{137} and NSAIDs are equally active in killing the COX deficient human colon cancer cell line HCT-15 as COX expressing colon cancer cell lines;\textsuperscript{138} a finding that is supported by recent experiments in COX-1 and COX-2 negative mouse fibroblasts.\textsuperscript{139}

If all NSAIDs are active in colon cancer regardless of COX inhibiting activity, this suggests that the effect is at least partly mediated through an alternative mechanism of action common to all NSAIDs. One obvious unifying feature of NSAIDs is that they are anti-inflammatory. Unrelated anti-inflammatory compounds such as Curcumin also show activity against colon cancer suggesting the importance of inflammation and of inflammatory molecules and pathways, in colon cancer.

Recent advances in the understanding of the molecular basis of inflammation reveal a central role for a relatively small number of intracellular pathways. These signal transduction pathways are well characterised and integrate the combined effects of large numbers of molecules at the cell surface to produce changes in the expression of a large number of genes. The NF-\(\kappa\)B, JNK, and p38 MAPK pathways are three important pathways involved with inflammation and we hoped that further understanding of their role in colon cancer would be revealing. These same molecules are also implicated in the regulation of apoptosis and proliferation of colonic epithelial cells.

There is also evidence linking NF-\(\kappa\)B and p38 MAPK more directly to the actions of NSAIDs, and linking JNK with colon cancer. Aspirin, Salicylic acid, Sulindac Sulphone and Curcumin all inhibit NF-\(\kappa\)B activation by inhibiting I Kappa B kinase.\textsuperscript{125, 126, 140, 115} The NF-\(\kappa\)B pathway is also involved in the activation of transcription of COX-2. This has led many to list NF-\(\kappa\)B as a COX-independent mechanism for the action of NSAIDs in colon cancer. Inhibition of p38 MAPK blocks the actions of Salicylates on colon cancer cells,\textsuperscript{127, 141} and JNK shows greatly enhanced activity in colon cancer in rats.\textsuperscript{130, 129}
In this study we set out to determine the site and degree of expression of the active forms of NF-κB, pJNK and p38 MAPK using immunohistochemistry in human sporadic colonic adenomas. Immunohistochemistry has the clear advantage of demonstrating which of the various cell types within the adenomas express these elements. Colonic adenomas were used because evidence has shown that these regress in patients treated with NSAIDs.\textsuperscript{32} Sporadic adenomas were chosen because they form the vast majority of colonic adenomas (>90%) and the magnitude of the reduction in colon cancer deaths attributed to NSAIDs (up to 50%) suggests that these precursors of colon cancer are likely to be NSAID sensitive.

We have demonstrated active NF-κB in interstitial cells in human colonic adenomas. Morphological analysis of the cells suggested that NF-κB localised to macrophages and this was confirmed using double staining techniques to show that the cells co-express CD68 antigen, a macrophage marker. We show that these same macrophages are also the site of expression of COX-2 and TNF-alpha. NSAIDs inhibit either COX-2 only (Indomethacin\textsuperscript{126}) or NF-κB only (Sulindac Sulphone\textsuperscript{140}) or both (Aspirin, Sulindac). These findings suggest that they could be acting on these distinct but related molecules in the same cells. We go on to show that p38 MAPK is active in macrophages in the stroma of polyps. There is evidence that p38 MAPK is involved in the activation of NF-κB in the cytoplasm\textsuperscript{142} so the finding of these two transcription factors both active in macrophages within polyps is not entirely unexpected.

The importance of macrophages in the progression of colon cancer has been suggested by other studies. COX-2 protein is expressed exclusively in intestinal macrophages in both normal colon and adenomas of Multiple Intestinal Neoplasia (MIN) mice but is not expressed at all in wild-type littermates.\textsuperscript{132} In human polyps localisation of COX-2 expression is more controversial. Initial reports showed COX-2 confined to neoplastic epithelial cells. However, two recent papers find that the highest expression is in stromal macrophages\textsuperscript{133, 134} although in advanced cancers expression seems to be in a far wider variety of cell types.\textsuperscript{143} There have been suggestions in the literature that the stromal staining is non-specific cross-reactivity of the polyclonal antibodies when used for immunohistochemistry.\textsuperscript{135} Our data confirm, using a different, monoclonal COX-2 antibody, that superficial stromal macrophages are the site of highest expression of COX-2. The finding that active NF-κB expression is highest in these same macrophages adds strong supporting evidence that the COX-2 staining is not non-specific antibody cross-reactivity, while also adding evidence for the specificity of the active NF-κB antibody.
The specificity of the antibodies used is obviously of paramount importance. We used TNF-alpha staining as further evidence for the specificity of the NF-κB staining because active NF-κB upregulates transcription of TNF-alpha and NF-κB is activated by TNF-alpha. We show that immunoreactivity of these two molecules is also seen in macrophages in colonic polyps. The same anti active NF-κB antibody has been used to perform immunohistochemistry in formalin fixed, paraffin embedded tissue in other studies including several that have shown activated NF-κB in macrophages.¹⁴⁴ ¹⁴⁵ We ourselves have previously shown the specificity of this antibody under the same conditions by showing co-localisation of TNF-alpha, TRAF-2 and total NF-κB staining with the active NF-κB staining and showing clear nuclear localisation of the active NF-κB.¹³⁶ Phosphospecific antibodies are also increasingly being used in immunohistochemistry in formalin fixed, paraffin embedded tissue, especially pJNK and pp38 MAPK.¹⁴⁶ ¹⁴⁷ Specificity is usually confirmed by showing results from immunoblot experiments. These have been performed extensively for the antibodies we have used including confirmatory kinase assays.¹⁴⁸ ¹⁴⁹

Higher numbers of infiltrating macrophages are associated with more advanced stage in colon cancer¹⁵⁰ and expression of the growth factor wnt that lies upstream of the APC/Beta Catenin pathway, the major pathway that is mutated in colon cancer, has also been localised to macrophages within the cancer stroma.¹⁵¹ The authors of these studies suggest that macrophages may have a paracrine effect possibly by suppressing the activity of other cytotoxic immune cells, or by the direct effect of the cytokines they secrete on the epithelial cells.

The importance of infiltrating lymphocytes in colon cancer has long been appreciated since higher numbers of infiltrating lymphocytes are associated with a more favourable outcome.¹⁵² ¹⁵³ This has led to extensive efforts towards immunotherapy for colon cancer. The finding of high expression of pJNK in T-lymphocytes infiltrating the polyp stroma is interesting because the kinase activity of JNK has been found to be dramatically increased in colon carcinomas.¹³⁰ JNK is one of the major kinases activating the transcription factor complex AP-1 that plays an important role in neoplastic transformation.¹⁵⁴

The importance of stromal cells in general is also receiving more attention. The stroma is felt to have reciprocal interactions with its overlying epithelial cells with each affecting the proliferation and differentiation of the other.¹³¹ Our findings of activation of central elements in pathways central to inflammation and carcinogenesis mainly confined to the stroma may add further credence to this hypothesis.