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Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit vascular smooth muscle cell proliferation via differential effects on the cell cycle

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Abstract

Abnormal vascular smooth muscle cell (VSMC) proliferation plays an important role in the pathogenesis of both atherosclerosis and restenosis. Recent studies suggest that high-dose salicylates, in addition to inhibiting cyclooxygenase activity, exert an antiproliferative effect on VSMC growth both in-vitro and in-vivo. However, whether all non-steroidal anti-inflammatory drugs (NSAIDs) exert similar antiproliferative effects on VSMCs, and do so via a common mechanism of action, remains to be shown. In this study, we demonstrate that the NSAIDs aspirin, sodium salicylate, diclofenac, ibuprofen, indometacin and sulindac induce a dose-dependent inhibition of proliferation in rat A10 VSMCs in the absence of significant cytotoxicity. Flow cytometric analyses showed that exposure of A10 cells to diclofenac, indometacin, ibuprofen and sulindac, in the presence of the mitotic inhibitor, nocodazole, led to a significant G0/G1 arrest. In contrast, the salicylates failed to induce a significant G1 arrest since flow cytometry profiles were not significantly different from control cells. Cyclin A levels were elevated, and hyperphosphorylated p107 was present at significant levels, in salicylate-treated A10 cells, consistent with a post-G1/S block, whereas cyclin A levels were low, and hypophosphorylated p107 was the dominant form, in cells treated with other NSAIDs consistent with a G1 arrest. The ubiquitously expressed cyclin-dependent kinase (CDK) inhibitors, p21 and p27, were increased in all NSAID-treated cells. Our results suggest that diclofenac, indometacin, ibuprofen and sulindac inhibit VSMC proliferation by arresting the cell cycle in the G1 phase, whereas the growth inhibitory effect of salicylates probably affects the late S and/or G2/M phases. Irrespective of mechanism, our results suggest that NSAIDs might be of benefit in the treatment of certain vasculoproliferative disorders.

Introduction

Abnormal vascular smooth muscle cell (VSMC) proliferation is a feature of a range of cardiovascular pathologies including atherosclerosis, restenosis following coronary angioplasty, in-stent stenosis and hypertension-induced vascular remodelling. In-stent stenosis, for example, has been estimated to affect over 100 000 patients worldwide (Mintz et al 1998) and has been shown almost exclusively to be due to the proliferation of neointimal VSMCs and their infiltration through the stent into the lumen of the vessel (Komatsu et al 1998). Thus, an understanding of the cellular mechanisms responsible for abnormal VSMC proliferation is important if we are to develop novel therapeutic regimes for in-stent stenosis and other vascular pathologies.

The non-steroidal anti-inflammatory drugs (NSAIDs) are a group of structurally diverse compounds that are used clinically for their analgesic and anti-inflammatory activities in the treatment of a range of conditions such as rheumatoid arthritis and osteoarthritis (Herfindal & Gourley 1996). They can also act as protective agents against cataract, the largest cause of blindness in the world (Blakytny & Harding 1992; Plater et al 1997). Several studies have reported that certain NSAIDs, such as sodium salicylate, sulindac and ibuprofen, when used at high doses can inhibit cell growth (Aas et al 1995; Marra et al 2000; Marra & Liao 2001; Tegeder et al 2001). Interestingly, the

anti-proliferative effects of high-dose NSAIDs have been shown to occur by a mechanism that is independent of their cyclooxygenase and prostaglandin synthesis inhibitory activities (reviewed in Tegeder et al 2001). Thus, Kopp & Ghosh (1994) demonstrated that high-dose sodium salicylate and aspirin inhibited the transcriptional activity of NF-κB in human Jurkat T cells and in the PD31 mouse pre-B cell line, whereas, in VSMCs, certain NSAIDs have been purported to inhibit cellular proliferation by regulating the expression and activity of components of the cell cycle machinery (Marra et al 2000; Marra & Liao 2001). However, whether NSAIDs of different structural classes are all capable of arresting VSMC growth, and whether they do so via a common mechanism of action, remains to be demonstrated.

In this study, we have used a series of NSAIDs, representative of different structural classes, including salicylates (aspirin and sodium salicylate), a phenylacetic acid derivative (diclofenac), a propionic acid derivative (ibuprofen), an indoleacetic acid derivative (indometacin) and a pyrroleacetic acid derivative (sulindac), to investigate their effects on the growth of rat A10 VSMCs. This cell line is particularly useful for investigating the potential effects of therapeutic agents in vasculoproliferative diseases, such as in-stent stenosis, since they resemble neointimal cells (Rao et al 1997). Our results show that, whereas all of the NSAIDs used in this study inhibited VSMC proliferation in a concentration-dependent manner, they exert their effects at different phases of the VSM cell cycle. Thus, the non-salicylate NSAIDs, diclofenac, ibuprofen, indometacin and sulindac, all block VSMCs at the G1 phase of the cell cycle, whereas salicylates induce a post-G1/S block and arrest cells in the G2 and/or M phases.

Materials and Methods

Materials

Unless specified, all chemicals and drugs used were of AnalaR grade and were obtained from Sigma Chemicals Ltd (UK). Dulbecco's Modified Eagle's medium (DMEM) with Glutamax, and fetal calf serum (FCS) were obtained from Gibco, Paisley, Scotland. Antibodies directed against cyclin A (sc-596), p107 (sc-318), $p21^{CIP1}$ (p21; sc-397) and $p27^{KIP1}$ (p27; sc-528) were obtained from Santa Cruz Biotechnology, Inc. (USA).

NSAID stock solutions

Sodium salicylate and the racemic compound, ibuprofen ($\pm$, sodium salt), were dissolved in phosphate-buffered saline (PBS); the prodrug, sulindac, was dissolved in PBS containing 10% dimethyl sulfoxide (DMSO); and acetylsalicylic acid (aspirin), indometacin and diclofenac (sodium salt) were dissolved in DMSO. All drugs were prepared as 1 M stock solutions (except aspirin, which was prepared as a 5 M stock solution) and were diluted directly into normal growth medium (DMEM/10% FCS) to give the appropriate working concentration.

Cell culture

Rat aortic A10 VSMCs were used in all studies and were maintained and passaged according to standard methods in DMEM supplemented with 10% FCS in a humidified atmosphere of 95% air/5% CO_2. For cell proliferation assays, cells were plated at a density of 1×10^4 cells/well in 6-well plates in the above medium. The following day, cells were re-fed with medium either lacking or containing various concentrations of the drug of interest. Seventy-two hours later, cells from each dish were trypsinized, pelleted and re-suspended in 6 mL Questor Isotonic Diluent (MicroMed Technical Ltd, Kent, UK) and the number of cells present in each dish determined in a Coulter Counter (Coulter Electronics, UK).

Flow cytometric analysis

The number of cells in G0/G1, S and G2/M was determined by flow cytometric analysis according to established methods (Li & Brooks 1997; Poolman et al 1998). Briefly, 2×10^5 cells were treated with or without NSAIDs for a total period of 48 h — a period of time sufficient to induce a significant cell cycle blockade in these cells (Harper & Brooks, unpublished results). During the final 24 h of the experiment, nocodazole (40 ng mL^{-1}), that blocks cells in the G2/M phases of the cell cycle and enables the effects of a G1 blocker to be evaluated more clearly, was added to the cultures containing NSAIDs. Thus, any NSAID(s) that caused a G1 block would not permit nocodazole-treated cells to progress through to G2. All cultures were incubated with 10 μM bromodeoxyuridine (BrdU) for the final 30 min of the experiment at 37 °C, then trypsinised, fixed in ice-cold 70% ethanol and stored at 4 °C until required for analysis. After removal of the ethanol, cells were digested with 0.1 M HCl in PBS for 10 min at 37 °C and BrdU detected with a mouse anti-BrdU monoclonal antibody (Becton Dickinson, UK) followed by incubation with rabbit anti-mouse F(ab)2-FITC antibody (DAKO, Denmark). Labelled cells then were incubated with PI staining solution (PI (50 μg mL^{-1}), RNAse A (200 μg mL^{-1}) in PBS) for 15 min before analysis on a FACScan flow cytometer (Becton Dickinson, UK). The percentage of cells in each phase of the cell cycle was calculated using the CellQuest software package (Becton Dickinson, UK).

Protein extraction and immunoblotting

Protein samples were prepared from control, untreated A10 VSMCs and cells treated with NSAIDs at various concentrations. Samples were prepared according to methods described previously (Brooks et al 1993, 1997). Proteins were separated electrophoretically on sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels and transferred to Hybond ECL nitrocellulose membranes (0.45 μm; Amersham, Pharmacia Biotech, UK) according to established methods (Brooks et al 1993, 1997). Membranes were incubated with rabbit polyclonal antibodies (1:1000 dilution) directed against cyclin A, p107, p21

and p27 and immunoreactive proteins detected according to methods described previously (Brooks et al 1993, 1997).

Protein determination

Protein concentrations in cell preparations were determined by the method of Bradford (1976) using BSA type V as a standard.

Cytotoxicity assay

Cells were plated at 1×10^4/well in 96-well culture plates in 200 μL of growth medium. The following day, cells were washed once with 100 μL PBS and then re-fed with phenol red-free DMEM containing 5% FCS, 0.4% glutamine, 110 mg L^{-1} sodium pyruvate and 4500 mg L^{-1} glucose with or without various concentrations of NSAIDs. Twenty-four hours later, lactate dehydrogenase (LDH) activity was determined in the medium as a measure of cytotoxicity in accordance with the manufacturer's instructions (Cytotox 96 Non-Radioactive Cytotoxicity Assay Kit (Promega)).

Statistical analysis

Quantitative data are shown as means ± standard deviations of the means unless otherwise stated. Differences in mean measurements between experimental groups were tested by analysis of variance followed by a *t*-test with a Bonferroni correction and differences were considered significant at the 95% level.

Results

NSAIDs inhibit VSMC proliferation in a non-cytotoxic manner

Treatment of A10 VSMCs with increasing concentrations of aspirin, sodium salicylate, diclofenac, ibuprofen, indometacin and sulindac produced a dose-dependent inhibition in cellular proliferation, as determined by whole cell counting, 72 h following drug treatment (Figure 1). The IC50 values of inhibition of VSMC proliferation (concentration of drug that inhibits growth by 50%) were determined from the dose–response curves and shown to be 3.32 mM for aspirin, 1.96 mM for sodium salicylate, 170 μM for diclofenac, 646 μM for ibuprofen, 331 μM for indometacin and 624 μM for sulindac. These values are considerably higher than those previously reported for inhibition of cyclooxygenase and prostaglandin synthesis by these drugs (Tegeder et al 2001). It should be noted that, since ibuprofen is a racemic mixture, comprising both *S*- and *R*-enantiomers, the above IC50 value for this compound reflects that for total *R*- and *S*- drug. That the inhibitory effects of these various NSAIDs on VSMC proliferation were induced via a specific non-cytotoxic mechanism was demonstrated by measuring the levels of LDH

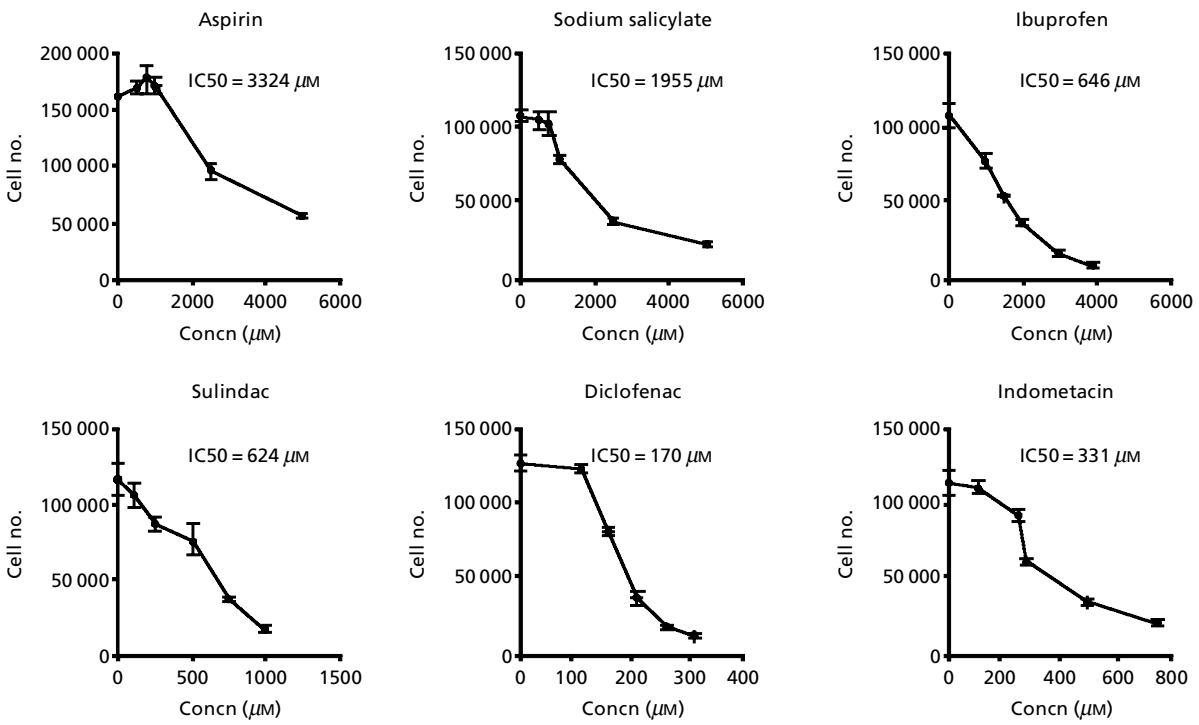

Figure 1 NSAIDs inhibit the proliferation of A10 VSMCs in a concentration-dependent manner. Cells (1×10^4 cells/well) were plated into 6-well tissue culture plates in DMEM containing 10% FCS. Twenty-four hours later cells were re-fed with medium containing various concentrations of NSAIDs. Cell numbers were counted in a Coulter counter 72 h later as described in Materials and Methods. Results show the mean ± s.d. values calculated from cell numbers present in triplicate wells per NSAID concentration. All experiments were performed at least twice.

enzyme released into the growth medium of cells treated with drug 24 h following treatment. Thus, even at the maximum drug concentrations used in these studies, LDH release, and therefore cytotoxicity, levels were minimal (less than 10% of control cells treated with vehicle alone; data not shown) suggesting that the growth inhibitory effects of NSAIDs in VSMCs were mediated by a specific mechanism.

Inhibition of the VSM cell cycle by NSAIDs

Previous reports in the literature have suggested that certain NSAIDs block VSMC proliferation by inhibiting the cell cycle machinery in these cells (Marra et al 2000; Marra & Liao 2001). However, a detailed analysis of the cell cycle profiles of NSAID-treated cells has not been reported. In addition, it remains unclear whether cell cycle arrest occurs at the same point in the cycle following treatment with all NSAIDs or whether different drugs cause arrest at different phases. To address these questions, we performed a series of flow cytometric analyses using the BrdU/PI double-staining method (Poolman et al 1998, 1999) to determine precisely where in the VSMC cycle each NSAID was eliciting its anti-proliferative effect. Since A10 VSMCs have an inherently high percentage of cells residing in the G1 phase of the cell cycle (Harper & Brooks unpublished observations), and because we hypothesised that at least some of these drugs would exert their effect during this phase of the cycle, we performed experiments in the presence of the mitotic inhibitor, nocodazole, that arrests cells in the G2/M phases and enables the effects of a G1 blocker to be evaluated more clearly. Thus, if a particular NSAID caused a G1 or S phase block, then cells would not proceed to the G2/M phases of the cell cycle in the presence of nocodazole but would be retained in the G1 or S phases (or both). Interestingly, when diclofenac, ibuprofen, indometacin and sulindac each were added to cultures of A10 VSMCs in the presence of nocodazole (40 ng mL^{-1}), a significant number of cells arrested in G1 and did not progress through S into the G2/M phases of the cycle (Figure 2 and Table 1). Thus, exposure of A10 cells to diclofenac, indometacin, ibuprofen and sulindac led to 33.8 ± 5.1%, 39.6 ± 1.9%, 24.2 ± 2.7% and 17.0 ± 0.8% of cells in G1, respectively, compared with 9.2 ± 1.8% in control cells treated with nocodazole alone, $P < 0.05$). Furthermore, the G1 arrest induced by these drugs occurred in the absence of a sub-G1 population of cells on the flow cytometric scan, suggesting that the effect occurred in the absence of apoptotic cell death consistent with the results of the cytotoxicity assay (see above and data not shown). In contrast, similar treatment of cells with anti-proliferative concentrations of the salicylate NSAIDs, aspirin and sodium salicylate, failed to induce a G1 block such that the percentage of cells found in the G2/M phases of the cell cycle was not significantly different from that found in cells treated with nocodazole alone (78.4 ± 3.6% and 77.1 ± 1.8% of cells in G2/M in aspirin and sodium salicylate treated cells, respectively, vs 80.2 ± 1.9% in control cells. See also Figure 2 and Table 1). These results suggest that aspirin and sodium salicylate inhibit VSMC proliferation after the G1/S border and most likely lead to a G2 or M phase block (or both).

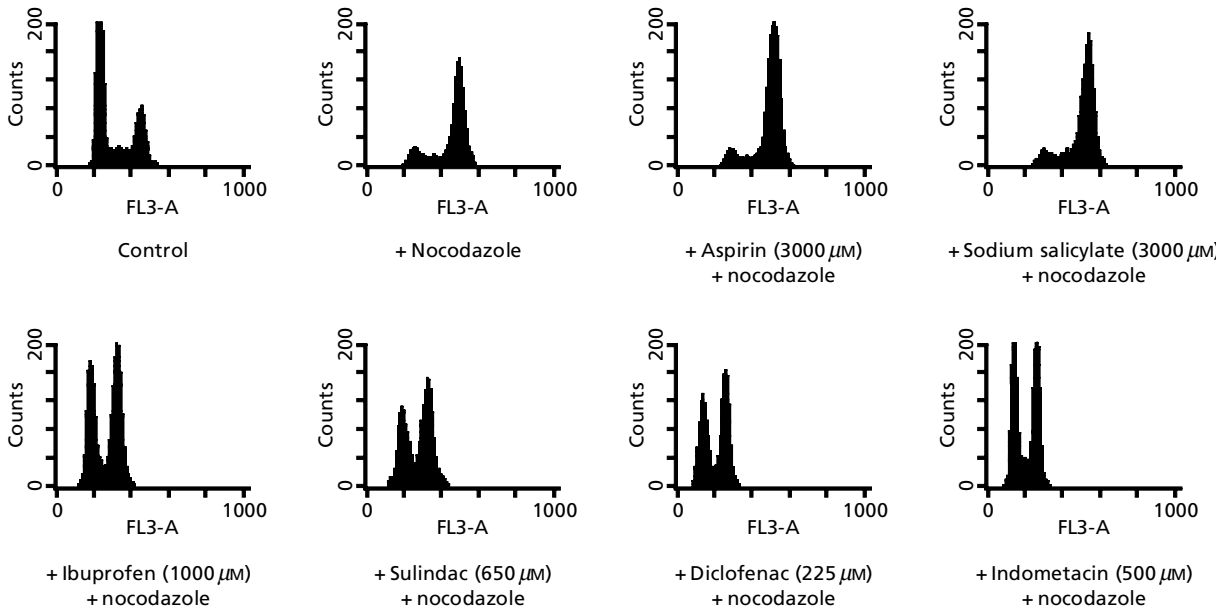

Figure 2 Non-salicylate NSAIDs inhibit A10 VSMC proliferation at the G1 phase of the cell cycle. Cells (2×10^5) were plated into 150-cm^2 flasks and treated 24 h later with NSAIDs at the indicated concentrations for a total period of 48 h. Where indicated, nocodazole was included at a final concentration of 40 ng mL^{-1}. Flow cytometric analysis was performed as detailed in Materials and Methods. Results show DNA histograms from a representative experiment. Similar results were obtained on at least two separate occasions.

Table 1 Inhibition of VSMC proliferation by different NSAIDs occurs at different stages of the cell cycle.

Treatment	G1	S	G2/M
Control	56.5 ± 3.1*	26.8 ± 1.7*	16.7 ± 0.7*
+ Nocodazole	9.2 ± 1.8	10.6 ± 2.5	80.2 ± 1.9
Aspirin	11.6 ± 4.0	10.0 ± 6.1	78.4 ± 3.6
Sodium salicylate	9.0 ± 2.1	13.9 ± 0.6	77.1 ± 1.8
Ibuprofen	24.2 ± 2.7*	7.8 ± 1.4	68.0 ± 2.7*
Sulindac	17.0 ± 0.8*	10.9 ± 1.7	72.1 ± 0.7*
Diclofenac	33.8 ± 5.1*	13.7 ± 0.6	52.5 ± 5.4*
Indometacin	39.6 ± 1.9*	21.1 ± 1.6*	39.3 ± 0.5*

Cells (2×10^5) were plated into 150-cm^2 flasks and treated 24 h later with NSAIDs at the same concentrations as used in Figure 2 for a total period of 48 h. Nocodazole was included at a final concentration of 40 ng mL^{-1} with NSAIDs as detailed in Figure 2. Flow cytometric analysis was performed as detailed in Materials and Methods. Results show means ± s.d. values for each stage of the cell cycle from three separate experiments; *$P < 0.05$, compared with nocodazole-treated cells (analysis of variance and Bonferroni t-test).

A previous report by Marra et al (2000) concluded that treatment of human VSMCs with salicylate NSAIDs led to a G1 arrest. These studies were conducted on quiescent G0 cells that were stimulated to re-enter the cell cycle with serum, whereas our studies were performed on asynchronous, growing cultures that more closely mimic the situation of VSMC proliferation during neointima formation. Therefore, we performed a separate series of experiments using A10 VSMCs that were synchronised in G0 by serum starvation for 48 h. Quiescent A10 cells then were stimulated to re-enter the cell cycle with 10% serum in the presence or absence of various NSAIDs. Nocodazole was also added to all cultures at the time of serum stimulation to ensure cells did not undergo more than one round of the cell cycle. Twenty-four hours after the addition of drugs, a flow cytometric analysis was performed to determine the percentage of cells in each phase of the cell cycle. Interestingly, the results of these studies were identical to those obtained with asynchronous A10 cells (Figure 2 and Table 1) thereby confirming that, whereas salicylate NSAIDs arrest the VSMC cell cycle, they do so after the G1/S transition. Taken together, our results suggest that, depending upon their chemical structure, NSAIDs can block VSMC proliferation by inhibiting either G1-dependent or G1-independent cell cycle mechanisms.

NSAIDs differentially affect expressions of specific cell cycle regulatory molecules

The flow cytometric data generated above suggests that, whereas certain NSAIDs inhibit VSMC proliferation in the G1 phase of the cell cycle, others do so independently of this phase of the cycle. To investigate the possible molecular mechanisms involved in the anti-proliferative effects of NSAIDs towards VSMCs, we determined how the expression levels of various cell cycle regulatory molecules were affected by NSAID treatment. A previous report in this field has shown that levels of the ubiquitously expressed cyclin-dependent kinase (CDK) inhibitors, p21 and p27, are up-regulated significantly following treatment with salicylate NSAIDs and that this was consistent with their inhibition of VSMC proliferation (Marra et al 2000). Indeed, we confirmed that, in our system, both p21 and p27 levels were elevated following treatment of VSMCs with salicylate and non-salicylate NSAIDs (Figure 3A). Interestingly, there was no discernible difference in the ability of salicylate and non-salicylate NSAIDs to induce expression of p21 despite an observable difference in the phase of the cell cycle that was inhibited by such treatment (Figure 2 and Table 1), although p27 levels appeared to be up-regulated in cells treated with non-salicylate NSAIDs compared with salicylate drugs. Since differences were observed in the ability of salicylate and non-salicylate NSAIDs to induce a G1 arrest in VSMCs, we next investigated whether the expressions of cell cycle regulatory molecules other than p21 and p27, that are known to act at specific stages of the cell cycle (Brooks & La Thangue 1999; Li & Brooks 1999), are modulated differentially by salicylate and non-salicylate NSAIDs. Thus, measurement of the relative levels of the G1/S phase regulatory molecule, p107, in drug-treated and control cells showed that the levels of hyperphosphorylated p107 were significantly higher in cells treated with aspirin and sodium salicylate than in those treated with the non-salicylate NSAIDs that showed higher levels of the faster migrating, hypophosphorylated form of p107 (Figure 3B). Non-treated A10 cells displayed a significant level of hyperphosphorylated p107, consistent with them being in a proliferative state (see Figures 2 and 3B). The phosphorylation status of p107 determines whether a cell can traverse the G1/S boundary or not, such that phosphorylation of this protein following activation of G1-acting cyclin–CDK complexes is required for normal cell cycle progression (Classon & Dyson 2001). Thus, our results are consistent with the non-salicylate NSAIDs arresting the VSMC cycle in the G1 phase, whereas salicylates induce cell cycle arrest via a G1-independent mechanism. We next determined the expression levels of the S/G2-acting protein, cyclin A, in drug-treated and control cells. Figure 3C shows that levels of cyclin A were elevated in salicylate-treated cells but not in non-salicylate treated cells. Taken together, these results are

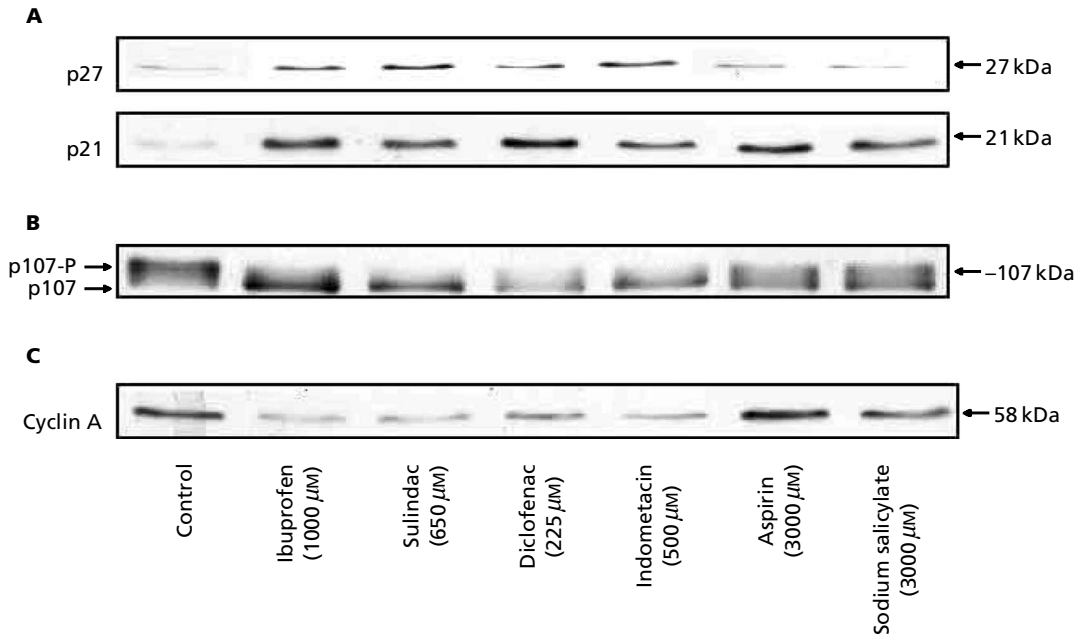

Figure 3 Anti-proliferative concentrations of salicylate and non-salicylate NSAIDs differentially affect the expression of G1, S and G2 phase-acting cell-cycle-regulatory molecules. A10 VSMCs were treated with similar concentrations of NSAIDs that were used in Figure 2 for a period of 48 h. Protein expressions of p21 and p27 CDK inhibitors (A), p107 (B) and cyclin A (C) then were determined by SDS-PAGE followed by immunoblotting as described in Materials and Methods. Total cellular protein (20 μg per lane) was loaded and equal transfer to nitrocellulose membranes confirmed by Ponceau S staining. The results shown are representative of three separate experiments. p107, hypophosphorylated p107; p107-P, hyperphosphorylated p107; control, cells maintained in DMEM/10% FCS.

consistent with the fact that diclofenac, ibuprofen, indometacin and sulindac induce VSMC arrest by blocking the G1 phase of the cell cycle whereas aspirin and sodium salicylate are likely do so via a late S and/or G2/M-phase arrest. It is feasible that p21 or p27 levels could be inducing the responses of all NSAIDs tested since these CDK inhibitors can block the activity of all cyclin–CDK complexes irrespective of the phase in the cell cycle at which they act (Brooks et al 1998).

Discussion

Restenosis and in-stent stenosis resulting from hyperproliferation of VSMCs and neointima formation following balloon angioplasty remain major clinical problems in the management of coronary artery disease (Isner et al 1996; Virmani & Farb 1999). We show in this study that whereas all NSAIDs tested inhibited VSMC growth in a concentration-dependent and non-cytotoxic manner, the non-salicylate drugs diclofenac, ibuprofen, indometacin and sulindac affected cell growth by blocking at the G1-phase of the cell cycle whereas the salicylates aspirin and sodium salicylate do so in a post-G1 manner that probably involves the late S or G2/M-phase cell cycle machinery.

Several recent studies have shown that certain NSAIDs, such as sodium salicylate, aspirin and sulindac, when used at high concentrations, can inhibit proliferation in a range of cell types, including certain tumour cells (Aas et al 1995) and VSMCs (Marra et al 2000). Indeed, the first indication that NSAIDs might have profound effects on cellular growth was reported by Waddell & Loughry (1983) who showed that sulindac could reduce the size of colonic polyps in patients suffering from Gardner's syndrome. Subsequent studies showed that regular aspirin use conferred a protective effect against colorectal cancer (Kune 2000) and in a randomised, double-blind controlled trial of 22 patients with familial adenomatous polyposis taking sulindac versus placebo showed that use of this NSAID led to a significant reduction in the number and size of colonic polyps (Giardiello et al 1993).

The fact that such drugs can inhibit cellular growth has prompted a recent investigation of their potential as therapeutic agents for the treatment of certain vasculoproliferative diseases (Reis et al 2000), such as restenosis and in-stent stenosis. Thus, Reis and colleagues demonstrated that sulindac, but not aspirin, significantly reduced neointimal formation following femoral artery balloon injury in both wild-type and apolipoprotein E-deficient mice, whereas both nitric-oxide-releasing aspirin (NCX-4016) and aspirin were effective in reducing restenosis in carotid artery balloon-injured LDL receptor-deficient mice (Napoli et al 2001). Marra et al (2000) subsequently showed that high doses of the salicylates aspirin and sodium salicylate inhibited human VSMC proliferation and hypothesised that this occurred as a result of a G1/S phase arrest on the basis that the expressions of certain cell cycle regulatory molecules were altered following drug treatment. Thus, the CDK inhibitors, p21 and p27, were

up-regulated following treatment of cells with salicylate NSAIDs and CDK2 activity was diminished (Marra et al 2000). While our results confirmed that these same NSAIDs inhibited rat VSMC growth, they failed to demonstrate that cell cycle arrest occurred in the G1 or S phases. Indeed, our flow cytometric analysis showed that both aspirin and sodium salicylate inhibited VSMC growth via a G1-independent mechanism. One reason for the discrepancy between the two studies likely relates to the fact that Marra and colleagues did not use flow cytometry to determine cell cycle profiles (Marra et al 2000), whereas we used the PI/BrdU double staining flow cytometric staining method that accurately defines the individual phases of the cycle (Poolman et al 1999). We confirmed that the salicylate NSAIDs did not induce a G1/S arrest by measuring the expression levels of various cell cycle regulatory molecules known to be expressed at various phases of the cell cycle (Brooks & La Thangue 1999; Li & Brooks 1999). Thus, the combination of the presence of hyperphosphorylated p107 and high levels of cyclin A suggest that aspirin and sodium salicylate block VSMC proliferation after the G1/S border.

Moreover, the absence of a significant percentage of salicylate-treated cells accumulating in the S phase, but that cells arrested in the G2/M phases, suggests that these NSAIDs inhibit VSMC proliferation within these latter phases of the cell cycle. Our observation that p21 and p27 protein levels were elevated following drug treatment is consistent with the study of Marra et al (2000). However, it is known that p21 and p27 are ubiquitous in terms of where they inhibit the cell cycle (Brooks et al 1998). Thus, an elevated level of these CDK inhibitors does not necessarily imply a G1 cell cycle arrest. Interestingly, it has been reported that salicylates inhibited primarily CDK2 activity (that is activated in the S and G2 phases of the cycle) and not CDK4/6 complexes (that are activated in G1) (Marra et al 2000), consistent with our observations that these drugs act at a later stage in the cell cycle than G1. We noted that the previous study was performed using synchronised, quiescent (G0) cells that were stimulated to re-enter the cell cycle with PDGF, whereas our initial experiments were performed on asynchronous, growing cultures that more closely mimic the situation of VSMC proliferation during neointima formation. To determine whether the differences between the two studies were due to the growth state of the cells, we performed a series of experiments on serum-starved A10 VSMCs that were stimulated to re-enter the cell cycle with serum in the presence or absence of NSAIDs. Inteestingly, we observed similar results in serum-starved A10 VSMCs treated with NSAIDs to those obtained with drug-treated asynchronous cultures such that aspirin and sodium salicylate caused a G2/M arrest whereas the non-salicyla te NSAIDs arrested cells at the G1/S border.

In addition to the observed effects on the cell cycle machinery, NSAIDs have been shown to affect other signalling pathways, most notably those modulated by NF-κB (Kopp & Ghosh 1994; Tegeder et al 2001). Indeed, Kopp & Ghosh (1994) showed that very high ($\sim$ 20 mM) concentrations of sodium salicylate and aspirin inhibited the transcriptional activity of NF-κB in human T-cells and in murine B-cells. Marra et al (2000) recently demonstrated that salicylate, but not indometacin, inhibits IκB kinase activity and partially blocks NF-κB activation and nuclear translocation in human VSMCs, implying a differential effect on intracellular signalling cascades for salicylate and non-salicylate NSAIDs.

Conclusion

Inhibiting components of the cell cycle machinery have been shown to be a useful therapeutic approach for treating a variety of vasculoproliferative diseases (Brooks & La Thangue 1999; Sriram & Patterson 2001). Thus, the possibility of using certain NSAIDs as a therapeutic strategy for inhibiting VSMC proliferation and neointimal formation following angioplasty, with or without a stent, appears to be a viable option. The pharmacokinetics of these drugs is well described and, despite the fact that the dose required for an anti-proliferative effect in cells is approximately 500- to 1000-fold higher than that required to inhibit cyclooxygenase activity and prostaglandin synthesis (Tegeder et al 2001), potential side effects such as gastric ulceration and bleeding would be minimised by the local and short-term administration of these drugs. Indeed, arthritic patients tolerate very high plasma levels of NSAID therapy and so the drug could be administered orally or be delivered via a non-oral, local route on a coated stent.

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