Induction of intercellular adhesion molecule 1 and class II histocompatibility antigens in colorectal tumour cells expressing activated *ras* oncogene

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Abstract

Aims—To determine whether there is a correlation between activation of the *ras* oncogene and the induction of MHC class II antigens and intercellular adhesion molecule 1 (ICAM-1) by interferon- γ (IFN- γ).

Methods—Expression of class II antigens, ICAM-1 and intracellular *ras* oncoprotein (p21) in established colorectal cell lines and short term cultures of primary colorectal tumour cells was determined by flow cytometry and mutation in the *ras* gene by sequencing of amplified segments of the gene.

Results—The cell lines showed a variation in their modulation of MHC class II antigens and ICAM-1, ranging from no induction to a 98-fold increase in class II antigen expression in the HT29 cell line. Previous work indicated that most tumours could not be induced to express class II antigens. Four of the five least inducible lines either contained mutant *ras* or highly expressed the oncoprotein. The four highly inducible cell lines all contained non-mutant *ras*. Of the 21 tumours studied in primary culture, 10 were inducible, one of which contained mutant *ras*. Of the remaining non-inducible tumours, four were mutant.

Conclusions—Correlations between *ras* activation and failure to respond to IFN- γ could not be shown to be significant. Therefore, *ras* activation, and concomitant subversion of intracellular signalling pathways, is probably not the major determinant in failure to activate class II antigens and ICAM-1.

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Inflammatory responses in tissues are often accompanied by augmentation of the expression of class II MHC antigens (HLA DR, DP and DQ) and of intercellular adhesion molecule 1 (ICAM-1) by cells. This is particularly apparent in rejection of allografts (for example, kidney¹) and in autoimmune disease (for example, inflammatory bowel disease²) and is ascribed to the production of cytokines, especially interferon- γ (IFN- γ), by activated T cells.

Inflammatory responses occur in a proportion of patients with cancer, with infiltration by leucocytes and upregulation at least of HLA DR. T cells responding in an MHC restricted manner to self antigens expressed by the tumours have been identified, suggesting that in some instances these inflammatory responses may be specific immune responses (reviewed in ³). There are some reports that a strong inflammatory response is indicative of a better prognosis (for example, in carcinoma of the larynx,⁴ breast,⁵ stomach,⁶ and colorectum⁷) and experimental data showing that expression of class II histocompatibility antigens by tumour cells influences their tumorigenicity.^{8,9} However, the extent of the inflammatory response in cancer is highly variable, with the degree of leucocytic infiltration ranging from virtually nil to very extensive, and the expression of class II antigens and ICAM-1 equally ranging from nil to very intense, with little correlation between the two. This variation may be related to differing capacities of tumour cells to respond to cytokines by upregulation of class II antigens and ICAM-1. We have demonstrated recently that tumour cells from most colorectal carcinomas which are negative for expression of MHC class II antigens and ICAM-1 are incapable of responding to IFN- γ by upregulating these molecules.¹⁰ In this paper we seek to demonstrate that this failure of response may be related to the activation of the proto-oncogene *Ki-ras* in the tumour cells, arguing that the subversion of intracellular signalling pathways resulting from such activation may disrupt cytokine responses.

The importance of the family of *ras* oncogenes is that it plays a central role in intracellular pathways, transducing growth factor signals from the cell membrane to the nucleus. The products of the *ras* oncogenes are small GTPases of 21 kDa (p21^{ras}) whose function is to transmit the signal from activated growth factor receptors to the *raf* gene product and thence via a series of mitogen activated kinases (MAP kinases) to the nucleus, where transcription of a range of genes important in cellular proliferation is stimulated.¹¹ Activation of *ras* by mutation or overexpression, or by subversion of other components of the pathway involving the *ras* gene product, results in stimulation of cell proliferation. In a number of human cancers there is activation of *ras* and in

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Table 1 Primers used for PCR amplification and sequencing

Primer identification	Primer sequence
Codon 12	
12.1B	5' GGC CTG CTG AAA ATC ACT GA 3'
12.2B	5' GTC CTG CAC CAG TAA TAT 3'
Codon 61	
61.1B	5' TTC CTA CAG GAA GCA AGT AG 3'
61.2B	5' CAC AAA GAA AGC CCT CCC CA 3'

particular in about 40% of cases of colorectal carcinoma.¹²

It has become clear recently that cytokines, in particular IFN- γ , act via a signalling pathway not directly involving p21^{ras}. The principal component of the IFN- γ signal transduction pathway is a protein with a molecular weight of 91 kDa which is activated by phosphorylation as a consequence of IFN- γ binding to its receptor. This protein then translocates to the nucleus where it activates transcription of a range of genes,¹³ including ICAM-1. In at least some cell types, however, IFN- γ signalling may involve additional mechanisms, particularly activation of protein kinase C (PKC).¹⁴⁻¹⁶

There is evidence that the different signal transduction pathways are not independent: "cross talk" occurs, with the activation of growth factor signalling pathways affecting cytokine responses and vice versa. In fact, it has long been established that cytokines influence cellular proliferation both positively (interleukin 2 and many others) and negatively (the IFNs and transforming growth factor- β). Equally, cell functions thought largely to be under the control of cytokines may be influenced by growth factor signalling pathways—for example, both epidermal growth factor and transforming growth factor suppress induction of class II antigens.¹⁷ The retinoblastoma protein, important in the control of cell cycling, can influence class II induction by IFN- γ .¹⁸ Activated *ras* can restore class II MHC antigen expression to class II negative B lymphoblastoid cells.¹⁹ Other oncogene products are known to influence class I MHC antigen expression—for example, *n-myc*²⁰ and adenovirus E1A protein.²¹ We have demonstrated that transfection of activated *ras* oncogenes into different murine fibroblasts results in both the transformation of the cells and changes in the expression of class II MHC antigens in response to IFN- γ .^{22,23}

"Cross talk" may occur at the level of PKC, which is activated both by growth factors²⁴ and by IFN- γ ¹⁴⁻¹⁶ and mediates at least some of the effects of both. Recently, it has argued that one of the crucial steps in the growth factor pathway, phosphorylation of the protein serine kinase *raf* (which initiates the MAP kinase cascade), requires activated PKC.²⁵ Indeed, it has been shown that activation of MAP kinase can be achieved by IFN- γ .¹⁸ It is also clear that growth factors binding to their receptors can result in the phosphorylation of p91, indicating another possible means of "cross talk".^{13,26-29}

We therefore hypothesised that in human cancer cells in which growth factor signalling pathways are subverted, cytokine responses may also be defective. In order to investigate

this, we have studied human tumour cells to determine whether subversion of the growth factor signal transduction pathway by *ras* activation influences the induction of expression of class II antigens and ICAM-1 in response to IFN- γ .

Methods

CELL LINES, CULTURE CONDITIONS AND PREPARATION OF PRIMARY TUMOUR CELL SUSPENSIONS

The following established colorectal cell lines: Caco-2, Colo201, Colo205, HCT116, HT29, LoVo, LS180, SW1417, and SW1417, were supplied by the American Type Culture Collection (ATCC Rockville, Maryland, USA) or the European Collection of Animal Cell Culture (ECACC: Public Health Laboratory Service, Porton Down, UK). Cell culture medium used was RPMI (Gibco BRL) supplemented with "Myoclon Plus" fetal calf serum (Gibco BRL). All incubation and culture procedures were carried out at 37°C in 5% CO₂.

To prepare tumour cell suspensions, the excised tumours were dissected to remove obvious necrotic tissue and blood clots, and were then washed in cold RPMI medium to remove surface contamination. The tumours were then minced, followed by enzymatic disaggregation with 0.01 mg/ml hyaluronidase type V (Sigma, Poole, Dorset, UK), 0.02 mg/ml DNase type I (Boehringer Mannheim, Mannheim, Germany), 1 mg/ml collagenase A (Boehringer Mannheim) in RPMI medium containing 10% "Myoclon Plus" serum by gentle shaking at room temperature for about 15 hours. The disaggregated tissue was then washed, and the tumour cells separated from stromal cells by centrifugation on Percoll (Sigma) gradients. The tumour cell fraction was then used to prepare DNA.

INDUCTION OF CLASS II MHC ANTIGENS AND ICAM-1 IN CELL LINES AND IMMUNOFLOUORESCENCE

Cells were incubated for three days with 10² units/ml of recombinant human IFN- γ , then trypsinised to prepare single cell suspensions, which were stained by direct immunofluorescence techniques using the monoclonal antibodies anti-ICAM-1 (CD54) clone 84H10 (fluorescein conjugate: Serotec MCA532, Oxford, UK) and L243 recognising a conserved HLA DR determinant (phycoerythrin conjugate: Becton Dickinson, Oxford, UK). The intensity of fluorescence was determined by flow cytometry using a Becton Dickinson FACStar.

PCR AND SEQUENCING

DNA was extracted by resuspending cell pellets in Hirt Buffer (0.01 M Tris/HCl (pH 7.4), 0.02 M EDTA, 0.5% (w/v) sodium dodecyl sulphate) at 10⁶ cells/ml and incubating with 100 g/ml proteinase K (Boehringer Mannheim) overnight at 37°C. The addition of a 1 in 30 volume of 3 M NaCl was followed by phenol/

chloroform extractions and the removal of contaminants by RNase/pronase (Sigma) treatments.

Because all *ras* mutations in colorectal tumours occur in codons 12/13 or 59/61 of the *Ki-ras* oncogene, these were the regions sequenced. These were amplified using the primers listed in table 1.

ras codon 12/13

PCR products were generated using the following cycle: one minute at 95°C, 30 seconds at 55°C, one minute at 72°C for 32 cycles, and then five minutes at 72°C. The 100 µl reaction mixture was made up of the following: 10 µl Taq buffer (500 mM KCl, 100 mM Tris/HCl (pH 9.0), 1% Triton X-100) (Promega), 2.5 mM MgCl₂, 80 ng DNA, 0.2 mM of each dNTP, 50 pmoles of each primer, and 2.5 units of Taq polymerase (Promega).

ras codon 61

PCR products were generated using incubations for one minute at 95°C, three minutes at 63°C for 32 cycles and then three minutes at 72°C. The 100 µl reaction mixture was made up of the following: 10 µl Taq buffer (500 mM KCl, 100 mM Tris (pH 9.0), 1% Triton X-100), 100 ng DNA, 0.2 mM of each dNTP, 2.5 mM MgCl₂, 100 pmoles of each primer, and 2.5 units of Taq polymerase. The PCR products were electrophoresed in Tris-acetate/EDTA buffer, excised from the agarose gel and purified by "Mermaid clean" (Bio 101) (for purification of fragments less than 200 base pairs in length) or by electroelution.

PCR products were sequenced by an adaptation of the Sequenase method, using the Sequenase 2.0 Version sequencing kit (US Biochemical Corporation). The 12.1B primer was used to sequence the *ras* codon 12/13, while the 61.1B primer was used to sequence the *ras* codon 61. Once sequencing was completed, the sequence was visualised by electrophoresis through a pre-run 6% acrylamide denaturing gel. The gel was run at 80 W for approximately two hours, then soaked in 10% glacial acetic acid for 35 minutes and dried at 80°C before being exposed to Kodak x ray film.

EXPRESSION OF p21^{ras}

The rat IgG₁ monoclonal antibody Y13-259 recognising the p21^{ras} protein encoded by the *Ki-ras* oncogene was prepared by harvesting culture supernatant from the hybridoma cell line CRL1742. The saturation dilution of the antibody of 1 in 200 was estimated by titration with a *ras* transfected mouse cell line. The rat antibody used as an isotype matched control reagent for the Y13-259 antibody was produced as above from the R46A2 hybridoma cell line (from E A Havell, Trudeau Institute Inc., New York, USA). As p21^{ras} is an internal protein, the staining procedure requires permeabilisation of the cells. Cells to be analysed were fixed in 2% paraformaldehyde in phosphate buffered saline (PBS) directly before use and incubated with

Table 2 Induction of class II antigen and ICAM-1 expression in colorectal cancer cell lines

Cell line	Class II antigen inducibility ¹	ICAM-1 inducibility ^{1,2}
HT	98.1	(+/-) 8.1
SW1417	78.2	(+) 6.2
Colo205	67.5	(+) 3.2
Colo201	11.6	(+/-) 3.3
HCT116	0→40*	(-) 0→3.9*
SW1463	0→21.8*	(+) 3.7
LoVo	2.2	(-) 1.5→4*
Caco-2	1.3	(++) 0
LS180	0	(+/-) 0→3.2*

¹ Inducibility is expressed as the ratio of the peak fluorescence of the induced cells to that of the uninduced cells—that is, the fold induction of the antigen. Where heterogeneous staining was apparent—that is, a broad peak of fluorescence: see fig 1B, indicated by *, the range of inducibility is given.

² Bracketed arithmetic signs indicate, in a semiquantitative manner, the extent of uninduced expression of ICAM-1.

the primary antibody diluted in 0.1% Tween 20 and 0.25% saponin at 4°C for one hour, followed by incubations of the biotinylated secondary antibody (Amersham International, Little Chalfont, UK) diluted 1 in 200 in diluent (0.1% Tween 20, 0.25% saponin, 10 mg/ml bovine serum albumin) for one hour at 4°C and Streptavidin/phycoerythrin complex (Amersham International) diluted 1 in 50 in diluent at 4°C for 45 minutes. Prior to analysis the cells were resuspended in PBS.

Results

EXPRESSION OF CLASS II ANTIGENS AND ICAM-1 BY TUMOUR CELL LINES

It was apparent that there was great variation in the expression of both class II antigens and ICAM-1 after treatment with IFN-γ and that the lines also showed heterogeneity of ICAM-1 expression in the absence of IFN-γ treatment. However, as all of the lines were essentially negative for class II antigen expression in the absence of IFN-γ treatment, it was possible to arrange the lines in order of inducibility for class II antigens: four lines (HT29, Colo205, SW1417, and Colo201) were all strongly inducible (fluorescence intensity more than 10-fold greater than without IFN-γ); LoVo was weakly inducible (less than twofold); two (Caco-2 and LS180) did not show significant inducibility, whilst two other lines (HCT116 and SW1463) showed heterogeneity of expression after treatment with IFN-γ.

Because of the constitutive expression of ICAM-1 by some of the lines, it is impossible to give an unequivocal order for its inducibility. However, there did seem to be a rough correlation between expression of the two, as the four lines which showed the most class II antigen induction were also relatively strongly inducible for ICAM-1, while three weakly inducible for class II antigens were also weakly inducible for ICAM-1. Of the two lines heterogeneous for induction of class II antigen expression, one showed strong ICAM-1 induction and the other was heterogeneous.

These data for class II antigen and ICAM-1 expression are summarised in table 2. Figure 1 shows flow cytometry profiles for the induction of class II antigens and ICAM-1 by representative cell lines (Caco-2, HCT116 and

Figure 1 Induction of class II antigen and ICAM-1 expression in colorectal cell lines. In all graphs the solid profile represents the background staining with control antibody. A, C and E, class II antigen staining; B, D and F, ICAM-1 staining. (A) Caco-2 class II trace VS19004 without IFN- γ and VS19005 with IFN- γ ; (B) Caco-2 ICAM-1 trace VS28003 without IFN- γ and VS28004 with IFN- γ ; (C) HCT116 class II trace VS8A008 without IFN- γ and VS8A026 with IFN- γ ; (D) HCT116 ICAM-1 trace VS27013 without IFN- γ and VS27014 with IFN- γ ; (E) HT29 class II trace VS8A006 without IFN- γ and VS8A005 with IFN- γ ; (F) HT29 ICAM-1 trace VS27009 without IFN- γ and VS27010 with IFN- γ .

HT29) which show contrasting responses to IFN- γ .

The inducibility of class I antigens in the cell lines was checked as a control of sensitivity to IFN- γ : all were inducible (data not shown). Non-enzymatic methods of cell removal were

compared with those incorporating trypsin to ensure that the trypsin did not significantly alter the levels of the expression, which was found to be the case (data not shown). In order to check that failure to express class II antigens or ICAM-1 was not the result of insufficient

Table 3 *ras p21 expression in colorectal cancer cell lines*

Cell line	Class II antigen inducibility rank ¹	ras specific fluorescence ³
Caco-2	8	705.3
LoVo	7	565.95
SW1417	2	442.45
HCT116	5 (m) ²	313.78
SW1463	6 (m)	241.16
LS180	9 (m)	198.13
HT29	1	158.9
Colo205	3	49.89
Colo201	4	44.0

¹As in table 2; ²m indicates mutant *ras*; ³peak mean fluorescence with background fluorescence subtracted.

IFN- γ , the length of incubation and concentration of IFN- γ was increased, with no effect (data not shown).

ACTIVATION OF *ras* IN TUMOUR CELL LINES

The *ras* gene may be activated either by mutation or by overexpression of the gene. Accordingly, we have sequenced the Ki-*ras* gene and measured p21^{ras} levels by immunofluorescence and flow cytometry in the cell lines described to determine their *ras* activation status.

Of the nine cell lines, three (HCT116, SW1463 and LS180) contained mutations in codons 12 or 13: these were HCT116 (codon 13: GGC→GAC); SW1463 (codon 12: GGT→TGT) and LS180 (codon 12: GGT→GAT). In the case of SW1463, the mutation was homogeneous (that is, no normal base was present), but in the other two cases there were normal bases present as well as mutant, indicating either heterozygosity or a mixed population. One of the lines (HCT116) showing heterogeneous class II antigen expression was sorted by fluorescence activated cell sorting to separate cells with high and low levels of expression. Of the sorted variants of HCT116, the non-class II expressing population seemed to contain the mutation, whilst the class II expressing population seemed to be normal. This suggests that the original population was a mixture of *ras* normal and mutant cells and it was the latter that failed to respond to IFN- γ .

The fluorescence data obtained for p21^{ras} are summarised in table 3 and allowed us to order the cell lines according to the level of expression of the gene: Caco-2>LoVo>SW1417>HCT116>SW1463>LS180>HT 29>Colo205>Colo201. Figure 2 shows representative flow cytometry profiles of p21^{ras} expression by the cell lines HT29, HCT116 and Caco-2.

INDUCTION OF COLORECTAL TUMOURS AND ACTIVATION OF *ras*

We have previously reported on the induction of MHC class II antigen and ICAM-1 expression by IFN- γ in tumour cells derived from colorectal carcinomas in primary culture.⁹ The extent of expression was determined by immunohistochemical techniques on cytospin preparations, identifying tumour cells by morphological criteria. The data indicated that most of the tumours could not be induced, but

that a few were inducible. In the latter class II antigen and ICAM-1 expression was induced in essentially all of the tumour cells. The data from 21 tumours are summarised in table 4.

The *ras* mutation status of these 21 tumours was determined by sequencing the Ki-*ras* gene. Of the 21 tumours, five harboured *ras* mutations, all of them in codon 12 (table 4). In all of these cases the normal base was also present, again suggesting heterozygosity. The corresponding normal colonic material collected

Figure 2 p21^{ras} expression by colorectal cell lines. (A) Caco-2, (B) HCT116 and (C) HT29.

Table 4 Class II MHC antigen and ICAM-1 inducibility and ras mutations in colorectal cancer cell lines

Tumour designation	Class II/ICAM-1 inducible	ras mutant	Codon 12/13 sequence ¹
VS 17	Yes	No	GGTGGC
VS 18	No	No	GGTGGC
VS 24	Yes	No	GGTGGC
ID 1	Yes	No	GGTGGC
ID 5	No	Yes	CGTGGC
ID 7	Yes	No	GGTGGC
ID 8	No	No	GGTGGC
ID 9	No	No	GGTGGC
ID 10	No	Yes	GATGGC
ID 11	Yes	Yes	TGTGCC
ID 12	Yes	No	GGTGGC
ID 13	Yes	No	GGTGGC
ID 14	No	No	GGTGGC
ID 19	Yes	No	GGTGGC
ID 20	Yes	No	GGTGGC
ID 28	No	Yes	CGTGGC
ID 29	No	No	GGTGGC
ID 33	Yes	No	GGTGGC
ID 36	No	No	GGTGGC
ID 37	No	No	GGTGGC
ID 41	No	Yes	GTTGGC

The mutant base is shown in bold.

at the same time as the primary tumour was also studied for *ras* activation. In all cases the normal colonic material was negative for the mutation in the corresponding tumour or indeed any other mutations (data not shown).

Flow cytometric techniques were unreliable because of the amount of debris present in the tumour cell suspensions and so we were unable to quantitate either surface antigen expression or p21^{ras} expression in the tumour cells by this technique.

Discussion

The response of tumours to cytokines is heterogeneous. We have investigated whether failure of tumour cells to respond to IFN- γ is because of subversion of growth factor signalling pathways in the tumour cells, particularly through *ras* activation. This was because, as we have discussed earlier, there is evidence for "cross talk" between growth factor and cytokine pathways. We have studied colorectal tumours as our experimental system because some 40% of these exhibit *ras* activation.

Of the cell lines studied, the most inducible all harboured normal Ki-*ras* genes and expressed low levels of p21^{ras}, suggesting that there is indeed a correlation between *ras* activation and inducibility. However, this cannot be shown to be statistically significant because of the difficulty in quantifying inducibility precisely in those cell lines where there was a range of expression. Of the 10 inducible tumours, with one exception, the *ras* gene was normal; of the 11 non-inducible tumours, four contained a mutant *ras* gene. However, χ^2 analysis revealed that the correlation was not significant.

These data clearly do not unequivocally support the hypothesis. Non-inducibility of cells with normal *ras* can be explained by adducing other possible mechanisms for subversion of the IFN- γ response, such as the inactivation of regulating proteins such as the GTPase activating protein NF-1 or activation of other growth factor receptors/tyrosine kinases, etc., in what is a complex system of overlapping components. Therefore, it is unlikely that there

will be a one to one relation between *ras* activation and failure of class II antigen/ICAM-1 induction.

Inducibility of cells from the tumour ID11 containing the GGT→TGT mutation, replacing Gly with Cys, is not so easily explained. However, there is evidence that not all mutations in *ras* affect its functioning equally. In an extensive series of experiments Seeburg *et al*³⁰ showed that the transforming ability of different codon 12 mutations varied with the amino acid substitution. It is also clear that codon 13 mutations affect the GTPase activity of p21^{ras} less than do codon 12 mutations.³¹ More recently, work by Finkelstein *et al*³² with colorectal carcinoma has suggested that the nature of the mutations of the *ras* gene could influence the biological behaviour of the cancer. In this instance the finding was that the particular substitution correlated with the degree of cancer spread. In particular, tumours with a Gly→Val substitution were indolent whilst those with a Gly→Asp mutation were very aggressive. Similar results have been obtained in pancreatic carcinoma.³³ It is therefore possible that the mutation occurring in ID11 (Gly→Val substitution at codon 12) which was IFN inducible was such that the IFN signalling pathway was less affected.

In summary, these data indicate that whilst *ras* activation may play a role in controlling responses to cytokines, clearly there are other important factors involved.

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