Familial polyposis coli: no evidence for increased sensitivity to mitomycin C

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SUMMARY Spontaneous chromosome instability is well established for the dominantly inherited cancer prone condition, familial polyposis coli (FPC), but conflicting results have been obtained regarding sensitivity to mitomycin C (MMC). We have investigated cell survival in fibroblasts and the induction of sister chromatid exchanges and chromosome damage in lymphocytes and fibroblasts after MMC treatment. We can find no evidence for a differential response of FPC cells as measured by any of these parameters, although individual FPC fibroblast cultures did show an enhanced chromosomal response. Overall, the FPC mutation does not appear to result in defective DNA repair in response to MMC.

Familial polyposis coli (FPC=adenomatosis of the colon and rectum) is an autosomal dominantly inherited condition. Affected subjects develop hundreds, possibly thousands, of adenomatous polyps in the colon and rectum. Without intervention, one or more progress to malignancy.¹ We and others have clearly shown spontaneously increased chromosome instability in all tissues examined from FPC patients.²⁻⁴ Following our initial observations, we have searched for evidence of differentially enhanced chromosome damage by clastogenic agents in parallel with investigations on cell survival. This communication reports our results using the anti-tumour antibiotic, mitomycin C (MMC).

Sasaki in 1978⁵ reported decreased cell survival following MMC treatment of skin fibroblasts from five FPC patients and one with Gardner's syndrome (that is, FPC with additional extracolonic lesions).⁶ Little *et al.*,⁷ using skin fibroblasts from two family members with Gardner's syndrome, found evidence for abnormal sensitivity to the lethal effects of X irradiation, ultraviolet light, and MMC. In contrast, Friedman *et al.*⁸ found an unexpected insensitivity in FPC fibroblasts to the induction of sister chromatid exchanges (SCEs) by MMC, although cultures from one of four patients exhibited lowered cell survival compared with control cultures. By 1983 Sasaki's group had investigated cells from nine FPC patients; about 50% were found to show increased susceptibility to

the cytotoxicity of MMC.⁹ Interestingly, the cultures hypersensitive to MMC were also hypersensitive to 4-nitroquinoline-1-oxide (4NQO). Most recently, Heim¹⁰ investigated the incidence of MMC induced gaps and breaks in lymphocytes in FPC and found no significant difference compared with controls.

We had done a considerable amount of work, both chromosomally and on cell survival, before we realised the full extent of the instability of MMC once in solution and on freezing and thawing, since noted by others.¹¹ Hence, all early work was discarded and we are reporting only data collected subsequently.

Materials and methods

With one exception, FPC patients were from the Polyposis Registry at St Mark's Hospital, London and have been described in an earlier publication.³ Patient FPC55 was obtained through Mr T Balfour at the City Hospital, Nottingham. Patients FPC5 and 22 are brothers, as are FPC7 and 9. Control material was from healthy volunteers. Skin fibroblast cultures were previously established in this laboratory³ with the exception of 48BR and 54BR which were obtained from Dr C Arlett (MRC Cell Mutation Unit, Sussex) as controls for Huntington's disease patients. All cultures were used between passages 6 and 21 and had been stored in liquid nitrogen; they were routinely tested¹² for mycoplasma contamination before and after the experiments with no positive results. Media and reagents were from Flow Laboratories unless otherwise stated.

MITOMYCIN C

Mitomycin C (Sigma) was dissolved in sterile deionised water to make a stock solution of 100 µg/ml. For the cell survival experiments this was aliquoted, frozen at -20°C, and a fresh bottle thawed for each experiment. For chromosome studies the solution was either used immediately or after overnight storage at 4°C. The solutions were always protected from light. Apart from loss of activity due to storage and repeated freezing and thawing, we also noted variation from batch to batch of the drug.

CELL SURVIVAL EXPERIMENTS

Medium used throughout consisted of Eagle's minimum essential medium (MEM) supplemented with 10% newborn calf serum and glutamine with 10 mmol/l HEPES buffer (Hopkin & Williams), penicillin (100 IU/ml), and streptomycin (100 µg/ml). The same batch of serum was used for all experiments. The colony forming assay was performed by plating treated cells onto feeder layers which were prepared as follows. Normal human fibroblasts in suspension, 5×10^5 cells/ml, were treated with MMC (5 µg/ml) for one hour at 37°C in a shaking water bath, washed once, and plated at 6×10^4 cells per 9 cm dish. After overnight incubation, the medium was aspirated off and replaced with fresh medium. Confluent flasks of test cells were incubated with 0.5 µg/ml MMC in medium for one hour, then brought into suspension, counted on a Coulter counter, and serially diluted. Control flasks were treated identically with omission of MMC. For each dilution 0.5 ml was added to each of five (for FPC patients) or 10 (for normal cultures) replicate feeder dishes. These were incubated in a humidified CO₂ atmosphere for 17 days, at the end of which time they were stained with Leishman's and the colonies counted. Survival was expressed as a percentage of colony forming cells compared with the number plated.

Colonies, consisting of at least 50 cells, were counted from plates with 80 to 100 colonies. Those from the next two dilutions were also counted, the numbers corrected for a dilution factor and the results combined by calculating a weighted mean. Plating efficiency was determined using untreated cells and expressed as colonies per number of cells plated.

CHROMOSOME STUDIES IN LYMPHOCYTES

Lymphocytes from patients and controls were cultured by standard methods using the same batches of medium and serum for both groups. Cells were harvested after 72 hours. For SCE estimation, BUdR was present at 5 µg/ml throughout and MMC

at 10 ng/ml for the final 24 hours.¹³ Thirty cells were scored from each of duplicate cultures per subject.

Chromosome damage was observed after treatment with MMC at 100 ng/ml for the final 24 hours. Gaps, breaks, and exchanges were recorded for 50 cells, from each of duplicate cultures. An aberration was scored as a gap if it did not exceed the width of a chromatid and there was no displacement of chromatin; an exchange was counted as two breaks. Damage was expressed as breaks per cell.

CHROMOSOME STUDIES IN SKIN FIBROBLASTS

Chromosomes were prepared by standard methods from rapidly growing cultures with a predominant normal karyotype. The exception was FPC3a, which was tetraploid.

For SCE estimation cells were cultured for 50 hours with BUdR at 2 µg/ml. MMC at 10 ng/ml was added for the final 24 hours. Where possible, 30 cells were scored per culture.

Chromosome damage was determined after treatment with 10 ng/ml MMC for 24 hours before harvesting. Fifty cells per subject were scored for gaps, breaks, and exchanges.

STATISTICAL ANALYSIS

Statistical analysis was carried out using the MINITAB computer package originally developed at the Penn State University. The Mann-Whitney two sample rank test was chosen as being the most suitable for the following reasons. (1) In some cases groups to be compared had significantly different variances, which excluded use of the pooled *t* test. (2) In other cases, groups had a variance of zero (all values in the group identical); this excluded use of the two sample *t* test. (3) The Mann-Whitney test is a powerful non-parametric test and it was thought better to be consistent and use this throughout.

Results

Cell survival after MMC treatment is shown in table 1. It can immediately be seen that the variability of response of the FPC cultures to the cytotoxic effects of the drug is matched (and exceeded) by that of the control cultures. The probability of the two groups being drawn from the same populations (by Mann-Whitney U test) is >0.5, that is, there is no significant difference.

SCE induction by MMC in lymphocytes is shown in table 2. For both patients and controls there was a significant increase in SCEs with treatment (FPC cultures, $p < 0.001$; control cultures, $p < 0.01$). However, both spontaneously and after treatment there was no significant difference in the SCE level between the two groups.

TABLE 1 Cell survival of skin fibroblasts after MMC treatment, 0.5 µg/ml for one hour.

Culture No	Passage No	MMC batch	Survival % (SE)	Plating efficiency % (SE)
Controls				
71LO	21	B	12.2 (0.3)	41.3 (0.9)
51LO	14	C	26.8 (0.8)	15.1 (0.4)
79LO	15	D	4.7 (0.2)	16.3 (0.4)
54BR	11	D	29.5 (0.9)	24.7 (0.6)
FPC				
3c	16	B	12.2 (0.4)	15.2 (0.6)
5d	12	B	9.0 (0.5)	18.8 (0.9)
5a	13	C	11.8 (0.3)	39.0 (1.0)
7c	12	C	20.4 (0.5)	64.6 (2.1)
8c	15	D	26.9 (1.4)	10.3 (0.3)

FPC5a and 5d are derived from separate primary cultures and separate biopsies.
For all tables, SE is standard error.

The extent of chromosome damage induced in lymphocytes can be seen in table 3. Since there was some variation with batch of the drug, the results are recorded separately. There appears to be slightly less damage induced in the FPC group of cultures than in controls, but there is in fact no significant difference.

The comparison of SCEs induced in FPC skin fibroblasts with those from controls is given in table 4. There is no significant difference in the mean basal SCE level per cell between the two groups of cultures. Similarly, with MMC treatment there is no significant difference in the means although the value for the FPC group is higher. This is principally owing to the very high value for FPC6c. For both groups there is a significant increase in SCEs with

treatment (FPC cultures, $p < 0.01$; control cultures, $p = < 0.05$).

Induced chromosome damage in fibroblasts is shown in table 5. Use of the monitor control culture 64LO indicated variation from batch to batch of MMC, so the results are given separately. 64LO was chosen for this role because it grew rapidly and chromosomes were easy to prepare, but it turned out to be an atypical control, as the values for MMC induced damage were far higher than for any other

TABLE 2 Sister chromatid exchanges induced in lymphocytes by MMC, 10 ng/ml for 24 hours.

	SCEs per cell	
	-MMC	+MMC
Controls		
1	8.2	12.6
2	5.3	8.6
3	5.6	14.4
4	6.1	9.8
5	7.3	11.1
7	8.4	14.0
8	5.3	12.2
	$\bar{X} = 6.6$ (0.49)	$\bar{X} = 11.8$ (0.79)
FPC		
4	4.2	10.6
14	5.6	13.9
32	5.3	12.7
18	6.3	10.4
19	5.9	8.7
20	6.0	9.1
3	5.1	9.6
21	4.4	12.0
22	3.9	8.6
	$\bar{X} = 5.2$ (0.3)	$\bar{X} = 10.6$ (0.63)

$\bar{X}$ =mean for the group (SE). 60 cells were scored per subject.

TABLE 3 Chromosome damage induced in lymphocytes by MMC, 100 ng/ml for 24 hours.

	Gaps+breaks/cell		Total abnormal cells (%)	
	-MMC	+MMC	-MMC	+MMC
MMC Batch I				
Controls				
1	0.02	0.24	2	19
2	0.04	0.24	3	21
3	0.01	0.24	1	18
	$\bar{X} = 0.023$ (0.009)	$\bar{X} = 0.24$ (0)		
FPC				
4	0.09	0.17	6	14
14	0.03	0.24	3	17
32	0.06	0.22	6	18
	$\bar{X} = 0.060$ (0.017)	$\bar{X} = 0.210$ (0.021)		
MMC Batch II				
Controls				
4	0.08	0.40	7	30
5	0.03	0.40	4	30
6	0.07	0.32	7	30
	$\bar{X} = 0.06$ (0.015)	$\bar{X} = 0.373$ (0.027)		
FPC				
18	0.04	0.30	7	26
19	0.05	0.30	5	24
20	0.03	0.32	3	22
	$\bar{X} = 0.04$ (0.006)	$\bar{X} = 0.307$ (0.007)		

100 cells were scored per subject.

$\bar{X}$ =mean for the group (SE).

control culture and resembled the highest FPC values.

Comparison of the mean number of induced gaps and breaks per cell for the FPC cultures with control cultures, including one mean value for 64LO, shows

TABLE 4 Sister chromatid exchanges induced in fibroblasts by MMC, 10 ng/ml for 24 hours.

	SCEs per cell	
	-MMC	+MMC
Control cultures		
51LO	6.5	10.0 (22 cells)*
64LO	5.3	25.5
71LO	5.0	25.2
73LO	6.2	22.1
78LO	6.4	17.0
	$\bar{X}$ = 5.88 (0.03)	$\bar{X}$ = 19.96 (2.92)
FPC cultures		
3a	11.4†	50.7†
5a	7.8	26.8 (22 cells)
6c	7.4	41.4
7c	5.7	16.0 (26 cells)
10d	6.8	11.7
55c	5.6	19.0 (15 cells)
	$\bar{X}$ = 6.50 (0.40)	$\bar{X}$ = 23.38 (4.28)

*30 cells were examined per culture except where indicated otherwise.

†Tetraploid karyotype, values halved in calculating means. $\bar{X}$ = mean for the group (SE).

TABLE 5 Chromosome damage induced in fibroblasts by MMC, 100 ng/ml for 24 hours.

		Gaps+breaks/cell	
		-MMC	+MMC
MMC Batch III	Control cultures		
	54BR	0.04	0.26
	64LO	0.04	0.50
	FPC cultures		
MMC Batch IV	5d	0.00	0.42
	Control cultures		
	48BR	0.09	0.16
	52LO	0.04	0.06
	64LO (i)*	0.14	0.30
	64LO (ii)	0.06	0.28
	77LO	0.02	0.06
		$\bar{X}$ = 0.06 (0.020)	$\bar{X}$ = 0.14 (0.055)
	FPC cultures		
	8c	0.06	0.28
MMC Batch V	9c	0.16	0.32
	10a	0.07	0.38
		$\bar{X}$ = 0.10 (0.032)	$\bar{X}$ = 0.33 (0.029)
	Control cultures		
	51LO	0.08	0.12
	71LO	0.00	0.02
	79LO	0.10	0.16
	81LO	0.04	0.18
		$\bar{X}$ = 0.055 (0.022)	$\bar{X}$ = 0.12 (0.036)
	FPC cultures		
	3c	0.04	0.12
	4f	0.14	0.24
	6c	0.02	0.14
	7c	0.04	0.10
		$\bar{X}$ = 0.06 (0.027)	$\bar{X}$ = 0.15 (0.031)

*64LO (i) and (ii) included twice to monitor the effect of overnight storage of MMC but one mean value used in calculations. $\bar{X}$ = mean for the group (SE). 50 cells scored per subject.

that there is no significant difference between the groups for MMC batches IV and V. Even if the results for 64LO are omitted as being non-representative of control cultures in general, the induced aberration rate in the FPC group is still not significantly higher than in the remaining controls.

The FPC cultures appear to fall into two groups: those from FPC4, 5, 8, 9, and 10 showing a high level of damage, whereas cells from FPC3, 6, and exhibit damage comparable to the controls excluding 64LO.

Discussion

In common with previous studies,^{5,8} we found considerable range of values for cell survival after MMC treatment for fibroblasts from people affected with FPC. Where our results differ from theirs is in the comparable range of values that we found for the controls. This is possibly because we have used a greater number of controls than some other authors. Another factor may be that all cultures were established from random donors and not obtained via a repository, where there may be selection of cell lines for inclusion. We thus have no evidence that FPC fibroblasts show increased sensitivity to the cytotoxic effects of MMC over and above that found for a variety of control fibroblasts.

Similarly, our data for both SCE induction and chromosome damage in lymphocytes show no differential response to MMC between affected and unaffected donors. These chromosome aberration results are in agreement with those of Heim,¹⁰ but we cannot agree with his proposition that this shows that there is no basic increase in chromosome instability in FPC lymphocytes. When looking for chromosome damage one is merely scoring the obvious aberrations and would miss the non-clonal rearrangements which made a large contribution to our observed increase in spontaneous chromosome type rearrangements in FPC cells³; detection of these depends upon full analysis.

In contrast to results by Friedman *et al.*,⁸ our data on SCE induction in FPC fibroblasts show a response equal to or above that for control cells. The apparent insensitivity of FPC cells to MMC induction of SCEs reported may have been the result of progressive loss of activity of the drug, as appears to have been repeatedly frozen and thawed during the course of the experiments. One of our fibroblast cultures, FPC6c, shows a very high response to MMC in terms of SCEs; this is of interest because at much later passage levels (about 30) this culture has repeatedly shown a spontaneous increase in chromosome rearrangements of all types such that the karyotype is grossly abnormal in at

cells of the culture (H Mazzullo, unpublished observations).

The data on chromosome aberrations induced in fibroblasts are difficult to interpret. There is obviously a difference in activity between batches of MMC, but it is impossible to decide by how much this affects the results. As stated, the FPC cultures appear to fall into two groups, of high and low responders in terms of MMC induced damage. However, the fact that cultures FPC7 and FPC9, derived from brothers, fall into different groups suggests that this grouping is not entirely related to the FPC gene. It may depend also on cell cycle differences at the time of exposure, as the cultures were only semi-synchronised (by subculturing from confluent flasks at the start of the experiment). The interpretation is further confounded by the repeatedly high response of the control culture 64LO. This was derived from an apparently normal 11 year old boy, but in addition to the high response of MMC induced damage these fibroblasts also respond to long term treatment with the tumour promoter, 12-0-tetradecanoyl phorbol-13-acetate (TPA) by repeatedly cloning in agar at high frequencies,¹⁴ suggesting that they are 'initiated' cells. This is an additional reason for considering 64LO to be somewhat different from the remaining eight control cultures. Since there is no proven increase in sensitivity to the cytotoxic effects of MMC, nor generally any differential increase in induced SCEs in lymphocytes or fibroblasts, it appears that people affected with FPC have no defect in DNA repair in response to this cross linking agent.

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