J Med Genet 1992; 29: 109–111

Cyclophosphamide increases the frequency of sister chromatid exchange in direct preparations of human chorionic villi in the absence of supplementary enzymatic activation systems

S Pariani, M Buscaglia, M Piantanida, G Simoni

Abstract

A study was performed to evaluate the effect on the frequency of sister chromatid exchange in first trimester chorionic villi of two chemical compounds, mitomycin C and cyclophosphamide. Mitomycin C is generally known to induce an increase in sister chromatid exchange both in vivo and in vitro standard conditions. Cyclophosphamide is a compound requiring additional enzymatic enrichment of the culture medium to express its mutagenic activity under in vitro conditions. We exposed chorionic villi samples to these chemicals without the use of conventional cell cultures and without adding enzymatic extracts to the medium. The results indicate a statistically significant increase in the frequency of sister chromatid exchange after exposure to both compounds and also at lower dosages.

Sister chromatid exchanges (SCEs) are considered to be sensitive indicators of genetic effects after exposure to mutagenic agents.[1–4] Cells treated with a variety of chemical and physical mutagenic and carcinogenic agents show an increased level of SCEs, both in vivo and in vitro conditions.[5] However, studies of chemically induced mutagenesis in vivo and in vitro differ greatly both in experimental techniques and conclusions.[6 7] Moreover it is necessary to make a distinction between indirect mutagens, such as cyclophosphamide (CP), and direct ones, such as mitomycin C (MMC).

Mitomycin C is generally recognised to induce an increased frequency of SCEs, both in vivo and in lymphocyte and skin fibroblast cultures.[8–10] However, cyclophosphamide, an antitumoural drug, shows mutagenic effect in vitro only after previous metabolic activation, normally present in vivo.[11 12] CP is metabolised by the liver yielding highly reactive alkylating compounds. Therefore, CP activation in vitro requires enrichment of culture systems with liver enzymatic extracts.

The alternative is to test the substance on a biological system that maintains sufficient enzymatic activity in vitro to permit expression of the substance itself. Recently, Carrera *et al*[13] tested the genetic effect of ultrasound using SCE analysis in spontaneous metaphases from chorionic villi, thus illustrating the possibility of investigating directly the effects of mutagenic agents in a human organised tissue structure without the need for cell culture.

In this paper, assuming the presumptive functional integrity of chorionic villi, we investigated their capacity to show not only the effects of direct mutagens, such as MMC, but also of chemicals requiring enzymatic activation, such as CP.

Materials and methods

Two series of experiments were performed. In the first series, three samples of first trimester chorionic villi obtained from voluntary termination of pregnancy were exposed to MMC. In the second, CP was tested on four samples of chorionic villi. In both experiments SCE frequencies were evaluated by comparing different concentrations of the agents to baseline conditions: MMC and CP were tested at 5 and 10 ng/ml and at 100 and 200 μg/ml final concentrations, respectively. CP was also compared to a positive control (5 ng/ml MMC).

VISUALISATION AND EXPOSURE OF SCEs

Chorionic villi sampled from 8 to 10 weeks voluntary abortions were selected under an inverted microscope (50 ×) and washed in Hank's balanced salt solution. Aliquots of about 20 mg were transferred into 35 mm plastic Petri dishes containing 3 ml of RPM-I 1640 medium with 5% fetal calf serum and antibiotics.[14] Villi were recovered in a humidified incubator with 5% CO_2 at 37°C for almost three to four hours before addition of 10 μg/ml BrdU and treatment with testing chemicals. MMC (Sigma), reconstituted and serially diluted in PBS without Ca++ and Mg++, was added to the dishes at 5 ng/ml and 10 ng/ml final concentrations. CP (Endoxan-Asta, Schering) was reconstituted in distilled water, diluted in Hank's balanced salt solution, and added to the dishes at 100 and 200 μg/ml final concentrations. Villi were then incubated for 72 hours and colcemid was added in a concentration of 0·08 μg/ml during the last three hours before harvesting.

Chromosome preparations were performed according to the procedure described by Simoni *et al*[15] and visualisation of SCEs was obtained by acridine orange staining.[16] The frequency of SCEs was evaluated in second division cells with no less than 44 chromosomes. The number of cells scored was either

Cattedra di Genetica Umana, Istituto di Scienze Biomediche, Ospedale San Paolo, Università di Milano, Via A di Rudini 8, 20142 – Milano, Italy.
S Pariani
G Simoni

Clinica Ostetrica e Ginecologica, Istituto di Scienze Biomediche, Ospedale San Paolo, Università di Milano, Milano, Italy.
M Buscaglia

Biologia Generale e Genetica Medica, Università di Pavia, Pavia, Italy.
M Piantanida

Correspondence to Professor Simoni.

Received 26 July 1991.
Accepted 5 August 1991.

Table 1 SCE frequency in second division metaphases after exposure to MMC of chorionic villi specimens obtained from three first trimester voluntary abortions.

Specimen	Control SCEs/cell		MMC 5 ng/ml SCEs/cell		MMC 10 ng/ml SCEs/cell	
	Average	Range	Average	Range	Average	Range
1*	3·6	(2–7)	10·2	(7–16)	17·7	(12–30)
2†	3·8	(1–8)	13·6	(10–20)	18	(14–24)
3*	3·45	(2–6)	12·24	(10–15)	16·3	(10–23)
	3·6		12		17·3	

Mann-Whitney Test
$C_{(1-3)}$ – MMC $5_{(1-3)}$ $p \ll 0{\cdot}01$.
MMC $5_{(1-3)}$ – MMC $10_{(1-3)}$ $p \ll 0{\cdot}01$.
No of metaphases analysed for each treatment: * 25, † 30.

Table 2 SCE frequency in second division metaphases after exposure to CP of chorionic villi specimens obtained from four first trimester voluntary abortions.

Specimen	Control SCEs/cell		Positive control* SCEs/cell		CP 100 μg/ml SCEs/cell		CP 200 μg/ml SCEs/cell	
	Average	Range	Average	Range	Average	Range	Average	Range
1†	3·8	(1–8)	13·5	(10–20)	12	(8–16)	—	—
2‡	3·6	(2–6)	12·5	(10–16)	10·6	(8–16)	17·4	(14–27)
3‡	3·4	(1–6)	13·2	(11–16)	12·7	(8–17)	24·4	(14–36)
4‡	3·04	(1–7)	11·5	(9–15)	10·2	(8–18)	17	(12–24)
	3·45		12·7		11·4		19·6	

Mann-Whitney Test
$C_{(1-4)}$ – CP $100_{(1-4)}$ $p \ll 0{\cdot}01$.
CP $100_{(1-4)}$ – CP $200_{(2-4)}$ $p \ll 0{\cdot}01$.
* Positive control: mytomicin C 5 ng/ml.
No of metaphases analysed for each treatment: ‡ 25, † 30.

25 or 30 for each treatment and specimen (table 1).

Results and discussion

MMC EXPOSURE

As summarised in table 1 a baseline value of 3·6 SCEs/cell was observed in control samples, while mean values of 12 and 17·3 SCEs/cell were observed after treatment with 5 and 10 ng/ml MMC, respectively. Statistical analysis, according to the Mann-Whitney test for large samples,[17] showed significant differences between the frequency of SCEs in exposed and control villi. In addition a significant difference was found when comparing the two concentrations of the compound.

CP EXPOSURE

The results of chorionic villi exposure to CP are shown in table 2. Chorionic villi showed their sensitivity to the presence of CP in the medium and the effect on the frequency of SCEs/cell increased with the concentration of the agent. In fact, after exposure of villi specimens to 100 and 200 μg/ml of CP, the frequency of SCEs/cell was 11·4 and 19·6 respectively, which was statistically significant when compared to 3·4 SCEs/cell baseline value. Moreover, a significant increase was found comparing the SCE frequency of the two dosages we tested. Exposure to 5 ng/ml MMC was used as a positive control and yielded a significantly higher frequency of SCEs than baseline value of unexposed villi.

It should be noted that the frequency of SCEs in untreated chorionic villi cells was found to be lower than that generally reported for human lymphocytes and fibroblasts.[8 18 19] In the present study, the statistically significant increase in SCEs occurred with a lower concentration of the two chemicals than used in previous reports on cultured cells.

All this might suggest that uncultured chorionic villi are very sensitive indicators of the mutagenic effect on chromosomes of chemical agents.

Conclusions

Chorionic villi appear to maintain a structural and functional integrity in vitro, sufficient to react to indirect mutagens without the addition of enzymatic extracts to the culture medium. In our study, samples of chorionic villi exposed to a direct mutagen, MMC, and to an indirect one, CP, showed a significant increase in SCEs/cell frequency compared to controls.

These results indicate that the mutagenic effect at the chromosomal level can be investigated without conventional cell culture. In fact, spontaneous cell proliferation of chorionic villi has been shown to be adequate for this purpose and indirect chemical mutagens requiring metabolic activation seem to be satisfactorily tested by this semi-in vivo system.

The authors would like to thank Professor M Fraccaro for helpful comments on the manuscript. This study was supported by grants from ASM, Associazione Italiana Studio delle Malformazioni, Milano.

1 Solomon E, Bobrow M. Sister chromatid exchanges – a sensitive assay of agents damaging human chromosomes. *Mutat Res* 1975;**30**:273–8.
2 Latt SA. Sister chromatid exchanges. *Genetics* 1979; **92**(suppl):83–95.
3 Wolff S, Carrano AV. Report of the workshop on the utility of sister chromatic exchanges. *Mutat Res* 1979;**64**:53–6.
4 Nakanishi Y, Schneider EL. In vivo sister chromatid exchanges: a sensitive measure of DNA damage. *Mutat Res* 1979;**60**:329–37.

5 Gebhart E. Sister chromatid exchange (SCE) and structural chromosomal aberration in mutagenicity testing. *Hum Genet* 1981;**58**:235–54.
6 Perry P, Evans HJ. Cytological detection of mutagen-carcinogen exposure by sister chromatid exchanges. *Nature* 1975;**258**:121–5.
7 Bochkov NP, Filippova TV, Kuzin SM, Stukalov SV. Cytogenetic effects of cyclophosphamide on human lymphocytes in vivo and in vitro. *Mutat Res* 1986;**159**:103–7.
8 Latt SA. Sister chromatid exchanges, indices of human damage and repair: detection by fluorescence and induction by mitomycin C. *Proc Natl Acad Sci USA* 1974;**71**:3162–6.
9 Huttner KM, Ruddle FH. Study of mitomycin C-induced chromosomal exchange. *Chromosoma* 1976;**56**:1–13.
10 Kram D, Schneider EL, Senula GC, Nakanishi Y. Spontaneous and mitomycin C-induced sister chromatid exchanges. Comparison of in vivo and in vitro systems. *Mutat Res* 1979;**60**:339–47.
11 Stetka DG, Wolff S. Sister chromatid exchange as an assay for genetic damage induced by mutagen-carcinogens. II. In vitro test for compounds requiring metabolic activation. *Mutat Res* 1976;**41**:343–50.
12 Lindhall-Kiessling K, Karlberg I, Olofsson AM. Induction of sister chromatid exchanges by direct and indirect mutagens in human lymphocytes, co-cultured with intact rat liver cells. Effect of enzyme induction and preservation of the liver cells by freezing in liquid nitrogen. *Mutat Res* 1989;**211**:77–87.
13 Carrera P, Orsini S, Terzoli G, Brambati B, Simoni G. Sister chromatid exchanges in first trimester chorionic villi after in vivo and in vitro exposure to diagnostic ultrasound. *Prenat Diagn* 1990;**10**:141–8.
14 Terzoli G, Romitti L, Guerneri S, Carrera P, Camurri L. Effect of incubation time and serum concentration on the number of mitoses in aspirated villi samples. In: Fraccaro M, Simoni G, Brambati B, eds. *First trimester fetal diagnosis*. Berlin: Springer-Verlag, 1985:197–200.
15 Simoni G, Brambati B, Danesino C, *et al*. Efficient direct chromosome analysis and enzyme determination from chorionic villi samples in the first trimester of pregnancy. *Hum Genet* 1983;**63**:349–57.
16 Dutrillaux MB, Laurent C, Couturier J, Lejeune J. Coloration des chromosomes humains par l'acridine orange après le traitment par le 5-bromodeoxiuridine. *CR Acad Sci [D]* 1973;**276**:3175–81.
17 Siegel S. *Non parametric statistics for the behavioral sciences*. New York: McGraw-Hill, 1956:116–27.
18 Mazzullo HA, Attwood J, Delhanty JDA. Familial polyposis coli: no evidence for increased sensitivity to mitomycin C. *J Med Genet* 1988;**25**:233–7.
19 Yokota K, Ueda K, Ohama K, Fujiwara A. Increased spontaneous and mitomycin C-induced SCEs in patients with cancer of the cervix uteri, with special reference to stage of cancer. *Cancer Genet Cytogenet* 1989;**43**:79–87.