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Protease inhibitors reduce the frequency of spontaneous chromosome abnormalities in cells from patients with Bloom syndrome

(cancer-prone/suppression of carcinogenesis/free radicals)

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ABSTRACT **Bloom syndrome is an autosomal recessive genetic disease. Cells from patients with this disease are characterized by high levels of chromosome aberrations and sister chromatid exchanges. We show here that the frequency of these chromosomal changes is markedly reduced when the cells are grown in the presence of certain protease inhibitors. In relation to other published data, our results suggest that the primary defect of Bloom syndrome cells may be related to the production of abnormally large amounts of agents, presumably active oxygen species, which are capable of acting like tumor promoters.**

Cells from people with the autosomal recessive genetic disease Bloom syndrome (BS) are characterized by a state of chromosomal instability; it is thought that the abnormally high level of chromosome aberrations and sister chromatid exchanges (SCE) observed in cells from people with this genetic disease in some way predisposes the affected individuals to a higher than normal incidence of cancer (1). Chromosome abnormalities, sometimes specific, are often associated with malignancies, as well as premalignant states; such chromosomal changes are likely to be an important step in carcinogenesis (1, 2). The experiments described here were performed to determine whether protease inhibitors could affect the spontaneous chromosome abnormalities occurring in cells from BS patients.

Certain protease inhibitors have been shown to prevent malignant transformation *in vitro* (3–6) and carcinogenesis *in vivo* (7–10) by physical and chemical carcinogens. The suppressive effect of protease inhibitors on carcinogenesis, first demonstrated in 1970 by Troll *et al.* (7), may be related to their action in blocking certain forms of DNA repair (11), although the evidence is conflicting on this point. (This hypothesis as well as other hypotheses to explain the nature of the protease inhibitor effects are discussed in ref 6.) Although the mechanism for the suppressive effect of protease inhibitors on carcinogenesis is presently unknown, protease inhibitors have been evaluated in our laboratory for possible use as human cancer chemopreventive agents.

In our experiments with BS cells reported here, three different protease inhibitors have been studied, antipain, soybean trypsin inhibitor, and the Bowman–Birk protease inhibitor from soybeans, all of which suppress carcinogen-induced transformation *in vitro* (3–6) and are possible cancer chemopreventive agents. Because antipain inhibits blood clotting and soybean trypsin inhibitor does not survive the gastric digestive process (Walter Troll, personal communication), these may not be the best possible cancer chemopreventive protease inhibitors. We have previously determined that the Bowman–Birk soybean protease inhibitor very effectively suppresses carcinogen-induced malignant transformation of cells *in vitro* and, when ingested in the diet, reaches the colon in an active form (5). Furthermore, there are some data which suggest that it may suppress carcinogenesis in experimental animals (8, 9). Thus, the Bowman–Birk protease inhibitor is a particularly promising human cancer chemopreventive agent. We report here that all three of the protease inhibitors we have studied are capable of suppressing chromosome abnormalities in BS cells.

MATERIALS AND METHODS

Two strains of BS cells were used in our studies: VS cells, established from a 6-year-old girl, were obtained from Michael Bender (Brookhaven National Laboratory, Upton, NY), and GM2548 cells were obtained from the Genetic Cell Repository at the Institute for Medical Research (Camden, NJ). As listed in the BS Registry (12), VS cells were derived from case 20 (ViSh) and GM2548 cells were derived from case 71 (HaEn). Three strains of normal human diploid fibroblasts were also obtained from the Genetic Cell Repository: AG1522 cells, which were derived from a 3-day-old infant foreskin sample, and the adult skin fibroblast cell strains GM3948 and GM3652 cells. All cells were used below passage 10 and were cultured at 37°C in a humidified 5% CO_2 in air atmosphere in minimum essential medium with Earle's salts, 25 μg of gentamycin per ml, and 15% fetal bovine serum. (Although the serum was not heat-inactivated in the studies reported here, we have performed other studies similar to those reported here with BS cells and protease inhibitors in which we used heat-inactivated serum. In these studies, the same three protease inhibitors as those utilized here reduced the levels of SCE and chromosome abberations in BS cells by ≈50%; thus, the results were similar to the data reported here.)

The protease inhibitors antipain (obtained from Walter Troll through the U.S.–Japan Co-operative Cancer Research Program) and soybean trypsin inhibitor (obtained from Sigma) were used at 25 μg/ml and 100 μg/ml, respectively. Two different preparations of the Bowman–Birk inhibitor were used; these were the purified inhibitor, prepared as described (5), and a crude extract of the inhibitor, described in detail (13) and prepared by Technion (Belleville, NJ). The crude extract contains five separate protease inhibitors, all of which are very similar to the Bowman–Birk inhibitor in molecular weight and protease inhibitor activity (14). The crude preparation is much less expensive and potentially of greater applicability in animal and human cancer chemoprevention studies, and, for these reasons, we have performed our experiments with both the pure and crude extract of the inhibitor. The protease inhibitor doses chosen were previously observed to be nontoxic and inhibitory to x-ray-in-

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Abbreviations: BS, Bloom syndrome; SCE, sister chromatid exchange(s).

duced malignant transformation of C3H10T½ cells *in vitro* (3–6). In a preliminary toxicity study, 25 μg of antipain, 100 μg of soybean trypsin inhibitor, and 50 μg of purified (5) Bowman–Birk inhibitor per ml were all shown to be nontoxic doses for the human cell strains used in the present experiments. The cells were maintained in the presence of the protease inhibitors for 2–3 weeks with at least one passage. When nearly confluent, the dishes were re-fed with culture medium containing the protease inhibitors. After 48 hr, the confluent, density-inhibited cultures were trypsinized and replated for chromosome aberration and SCE analyses. Initially, this last stage was carried out in the presence of protease inhibitors, but in later experiments, to conserve protease inhibitors, they were not present in cultures being prepared for the chromosome and SCE analyses (no difference in our results was observed with this change in experimental protocol).

The techniques for measuring chromosome aberrations and SCE have been described elsewhere (15, 16). Briefly, the cells were subcultured from confluent, stationary-phase cultures into four T-30 flasks at a density of $\approx 3 \times 10^5$ cells per flask in medium (in experiments 2 and 4–6, shown in Table 1, the medium also contained BrdUrd at a final concentration of 10 μM so that an analysis of SCE could be performed at the same time as the chromosome aberration anal-

Table 1. Effects of protease inhibitors on chromosome aberrations and SCE in normal human diploid cells (1522, GM3948, and GM3652) and cells from BS patients (VS and 2548)

			Chromosome aberration analysis							
					Chromatid-type		Chromosome-type			
Treatment group	Exp. no.*	SCE analysis (total SCE/total chromosomes)	Total number of cells scored	Total number of chromosome aberrations (−gaps)	Breaks	Exchanges	Breaks	Rings and dicentrics	Gaps	Total number of chromosome aberrations (−gaps)/total number of cells scored
Normal cells (controls)										
1522 cells										
No treatment	1	—	53	1	1	0	0	0	1	
	2	209/2476 = 0.08	53	1	1	0	0	0	1	
+ Antipain	1	—	53	0	0	0	0	0	0	6/318 = 0.02
	2	—	53	2	1	0	1	0	1	
+ Soybean trypsin	1	—	53	0	0	0	0	0	0	
inhibitor	2	—	53	2	1	0	1	0	1	
GM3948 cells										
No treatment	5	178/1609 = 0.11	100	1	1	0	0	0	4	
+ Antipain	5	128/1165 = 0.11	53	0	0	0	0	0	0	
+ Soybean trypsin inhibitor	5	134/1359 = 0.10	49	0	0	0	0	0	0	4/356 = 0.01
+ Crude Bowman–Birk inhibitor	5	66/772 = 0.09	154	3	3	0	0	0	2	
GM3652 cells										
No treatment	6	218/1820 = 0.12	53	2	2	0	0	0	0	
+ Antipain	6	74/903 = 0.08	53	1	1	0	0	0	1	
+ Soybean trypsin inhibitor	6	99/898 = 0.11	53	2	2	0	0	0	0	6/212 = 0.03
+ Crude Bowman–Birk inhibitor	6	84/872 = 0.10	53	1	1	0	0	0	2	
BS cells										
VS cells										
No treatment	5	1087/972 = 1.12	159	28	18	0	8	2	12	34/212 = 0.16
	6	1124/1133 = 0.99	53	6	4	1	1	0	4	
+ Antipain	5	551/929 = 0.59	53	5	3	0	1	1	2	9/106 = 0.08
	6	744/1297 = 0.57	53	4	3	0	1	0	3	
+ Soybean trypsin	5	947/1701 = 0.56	53	4	3	0	1	0	1	9/106 = 0.08
inhibitor	6	581/912 = 0.64	53	5	4	1	0	0	1	
+ Crude Bowman–Birk inhibitor	5	1532/2298 = 0.67	53	5	3	0	1	1	3	8/106 = 0.08
	6	674/1162 = 0.58	53	3	1	0	1	1	1	
2548 cells										
No treatment	2	2819/2568 = 1.10	106	7	3	0	3	1	2	15/212 = 0.07
	3	—	106	8	5	0	3	0	2	
	4	1678/1630 = 1.02								
+ Antipain	2	1844/2504 = 0.74	106	3	3	0	0	0	0	6/212 = 0.03
	3	—	106	3	2	0	1	0	0	
	4	606/847 = 0.72								
+ Soybean trypsin	2	1019/1527 = 0.67	106	2	2	0	0	0	1	2/159 = 0.01
inhibitor	3	—	53	0	0	0	0	0	1	
	4	700/1112 = 0.63								
+ Purified Bowman–Birk inhibitor	2	987/1394 = 0.71	106	5	5	0	0	0	2	11/265 = 0.04
	3	—	159	6	5	0	1	0	3	

*For the chromosome aberration analysis, experiments 1 and 3 were performed without the presence of BrdUrd; experiments 2 and 4–6 were performed with BrdUrd so that the SCE analysis could be performed simultaneously with the chromosome aberration analysis.

ysis). Colchicine (2 μM) was added to one of the flasks at 6-hr intervals beginning 28 hr after subculture to arrest cells in metaphase during the first mitosis after subculture. The cells were fixed by the hypotonic method and stained with 2% acetoorcein (17). About 50–150 metaphase figures were analyzed from the sample containing the peak mitotic index for each experimental point.

For SCE, the cells were cultured with 10 μM BrdUrd in complete medium for two rounds of cell replication (40–50 hr) beginning immediately after subculture. Colchicine (2 μM) was added to successive samples for 4-hr intervals before fixation as described in detail elsewhere (15). The cells were fixed by the same procedure as used for identifying chromosome aberrations. The chromosomes were stained by the fluorescence plus Giemsa technique (18) for the differential staining of sister chromatids as described (15). SCE were scored in the sample containing the peak number of second mitoses.

RESULTS

The results of our studies on the effects of three protease inhibitors on SCE and chromosome aberrations in three strains of normal cells and two strains of BS cells are shown in Tables 1 and 2. The cells from two BS patients had significantly higher frequencies of spontaneous SCE and chromosome aberrations than normal cell strains. Both SCE and chromosome aberrations in the BS cells were significantly reduced by protease inhibitor treatment, although not to the low levels observed in normal cells. We observed that antipain, soybean trypsin inhibitor, the purified Bowman–Birk inhibitor, and the crude extract of the Bowman–Birk inhibitor all had similar suppressive effects on the frequencies of SCE and chromosome aberrations in BS cells. For the three normal cell strains studied, protease inhibitors did not have significant effects on the very low levels of spontaneously occurring SCE and chromosome aberrations.

The question arose as to whether the observed reduction in the levels of chromosome abnormalities in BS cells by protease inhibitors could be due to mitotic delay of a subpopulation of BS cells having high levels of chromosome abnormalities such that these cells would not have been included in the chromosome analyses performed. To determine whether protease inhibitors affect the cell cycle, we have performed a continuous labeling index study using VS and normal (1522) cells cultured either with or without the presence of antipain, soybean trypsin inhibitor, or the crude extract of the Bowman–Birk inhibitor (at the concentrations used for the chromosome abnormality studies) for 2 weeks prior to the addition of [^{3}H]thymidine to cultures. Protease inhibitor treatment was maintained in some of the cultures while the protease inhibitors were removed from other cultures during the 4-day period of the labeling index study; these cultures were then compared to control cultures of normal or BS cells that had never been exposed to protease inhibitors. In this study, we observed that the percentage of labeled cells at each time point was approximately the same for VS or normal cells either with or without the presence of protease inhibitors for any of the time periods studied (with ≈90% of the BS cells being labeled by day 2, at which time a plateau level for the labeling index was reached in all of the cell populations studied). Thus, these results suggest that the protease inhibitors used in our studies do not affect the cell cycle of either VS or 1522 cells.

DISCUSSION

It is thought that the high levels of chromosome abnormalities in BS cells are related to the high incidence of cancer in BS patients (1). There is now evidence to suggest that protease inhibitors can suppress carcinogenesis induced by physical and chemical carcinogens, as discussed in the Introduction. Given the present results suggesting that protease inhibitors reduce the levels of spontaneous chromosome abnormalities in BS cells, it is possible that they could also suppress the spontaneous cancer incidence in BS patients.

We report that the protease inhibitors antipain, soybean trypsin inhibitor, and both a purified form and a crude extract of the Bowman–Birk inhibitor can reduce the abnormally high level of both chromosome aberrations and SCE in BS cells by ≈50%. Although it is not known why the level of chromosome abnormalities was not reduced further by the protease inhibitors to the low levels observed in the normal cell strains studied, it is possible that longer incubation times with protease inhibitors could have greater effects than those reported here. We do know that concentrations of protease inhibitors that are higher than those utilized in the studies reported here (300 μg/ml for the Bowman–Birk inhibitor and 50 μg/ml for antipain) do not result in a greater effect. These results suggest that the observed effect has been saturated and that protease inhibitors may only be able to affect 50% of the process involved in the production of high levels of SCE and chromosome aberrations in BS cells.

The mechanism for the protease inhibitor reduction of chromosome abnormalities in BS cells is not clear; however, there are several possible mechanisms for the observed effect. It is conceivable that in the presence of protease inhibitors, pre-existing cells with high levels of chromosome abnormalities are not proliferating normally with the rest of the population of BS cells and would thus not have been included in the chromosome analyses performed. Our results showing that the three protease inhibitors used in our studies do not affect the cell cycle of either normal or BS cells argues against this possible mechanism and suggest that the observed effect of protease inhibitors on chromosome abnormalities is not related to a cell selection phenomenon due to mitotic delay among a subpopulation of the cells.

The most likely explanation for our results is that *de novo* formation of aberrations and SCE is prevented in the presence of protease inhibitors. Emerit and co-workers (19, 20)

Table 2. χ^2 analyses to compare the results for the various groups

Treatment group	SCE analysis: Total SCE/total chromosomes	SCE analysis: χ^2	Chromosome aberration analysis: Total number of chromosome aberrations (−gaps)/total number of cells scored	Chromosome aberration analysis: χ^2
BS cells	6,708/6,303 (1.064)	523, $P < 0.001$*	49/424 (0.116)	18.8. $P < 0.001$*
BS cells with protease inhibitors†	10,185/15,683 (0.649)	2117, $P < 0.001$‡	45/954 (0.047)	3.8, $P = 0.05$‡
Normal cells	605/5,905 (0.102)	0.5, $P = 0.5$§	5/259 (0.019)	0.03, $P = 0.85$§
Normal cells with protease inhibitors	585/5,969 (0.098)		11/627 (0.018)	

*BS cells vs. BS cells with protease inhibitors.
†When compared to BS cells without protease inhibitors and tested individually, each of the three protease inhibitors showed a highly significant inhibition of both SCE and chromosome aberrations.
‡BS cells with protease inhibitors vs. normal cells.
§Normal cells vs. normal cells with protease inhibitors.

have reported that a clastogenic factor is present in cultured BS fibroblasts and in the plasma of BS patients, and they have observed that superoxide dismutase inhibits the effect of this factor (20). Because free radicals are known to induce SCE (21) as well as chromosomal aberrations, Emerit and Cerutti (20) have hypothesized that cells from BS patients may be deficient in the detoxification of active oxygen species. Alternatively, Cerutti (22) has suggested that BS cells may possess an abnormality in the formation of active oxygen species. Although there is no evidence that protease inhibitors interact with, or detoxify, free radicals, it has been reported that protease inhibitors can prevent the production of the superoxide anion radical ($O_2^- \cdot$) and hydrogen peroxide that follows exposure of human polymorphonuclear leukocytes to tumor-promoting agents (23). Our finding that protease inhibitors reduce the levels of spontaneous chromosome abnormalities in BS cells suggests, therefore, that the primary defect of these cells is the production of abnormally large amounts of agents capable of acting like tumor promoters, presumably active oxygen species, rather than that they fail to detoxify them. It is possible that the abnormalities shown by these cells are due either to spontaneous occurrence of the reaction that is stimulated by promoters or to extreme sensitivity to low levels of tumor promoters present in the cells' environment.

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