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Progesterone Induces BRCA1 mRNA Decrease, Cell Cycle Alterations and Apoptosis in the MCF7 Breast Cancer Cell Line

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Abstract. *Background: Inherited mutations of the BRCA1 gene are responsible for hereditary breast and ovarian cancer syndrome. However, little is known of how disruption of BRCA1 functions preferentially increases cancer risk in hormone-dependent organs. We aimed to study whether BRCA1 was regulated by progesterone in the MCF7 breast cancer cell line. Materials and Methods: MCF7 breast cancer cells were incubated with 10^{-4} or 10^{-10} M progesterone for 24 or 48 hours. BRCA1 expression, proliferation and apoptosis were analysed. Results: 10^{-4} M progesterone decreased cell proliferation, cell cycle progression and induced apoptosis. In addition, BRCA1 and cyclin A mRNA decreased. In contrast, none of these effects were observed in MCF7 cells incubated with 10^{-10} M progesterone. Conclusion: The down-regulation of BRCA1 in MCF7 cells incubated with 10^{-4} M progesterone seems to be a consequence of cell cycle alterations rather than a direct effect of the hormone on BRCA1.*

BRCA1 is a tumor suppressor gene that is responsible for hereditary breast and ovarian cancer syndrome (1). Women inheriting mutations of BRCA1, most of which are truncating mutations that result in non-functional or unstable proteins (2), have an 85% likelihood of developing breast cancer in their lifetime (3) and have a poorer prognosis compared to sporadic breast cancer (4-6). Although somatic mutation of BRCA1 is a rare event in sporadic tumors (7, 8), a decreased expression of BRCA1 was reported in high-grade non-inherited breast cancers, which suggests that BRCA1 may also play a role in sporadic breast cancer (9).

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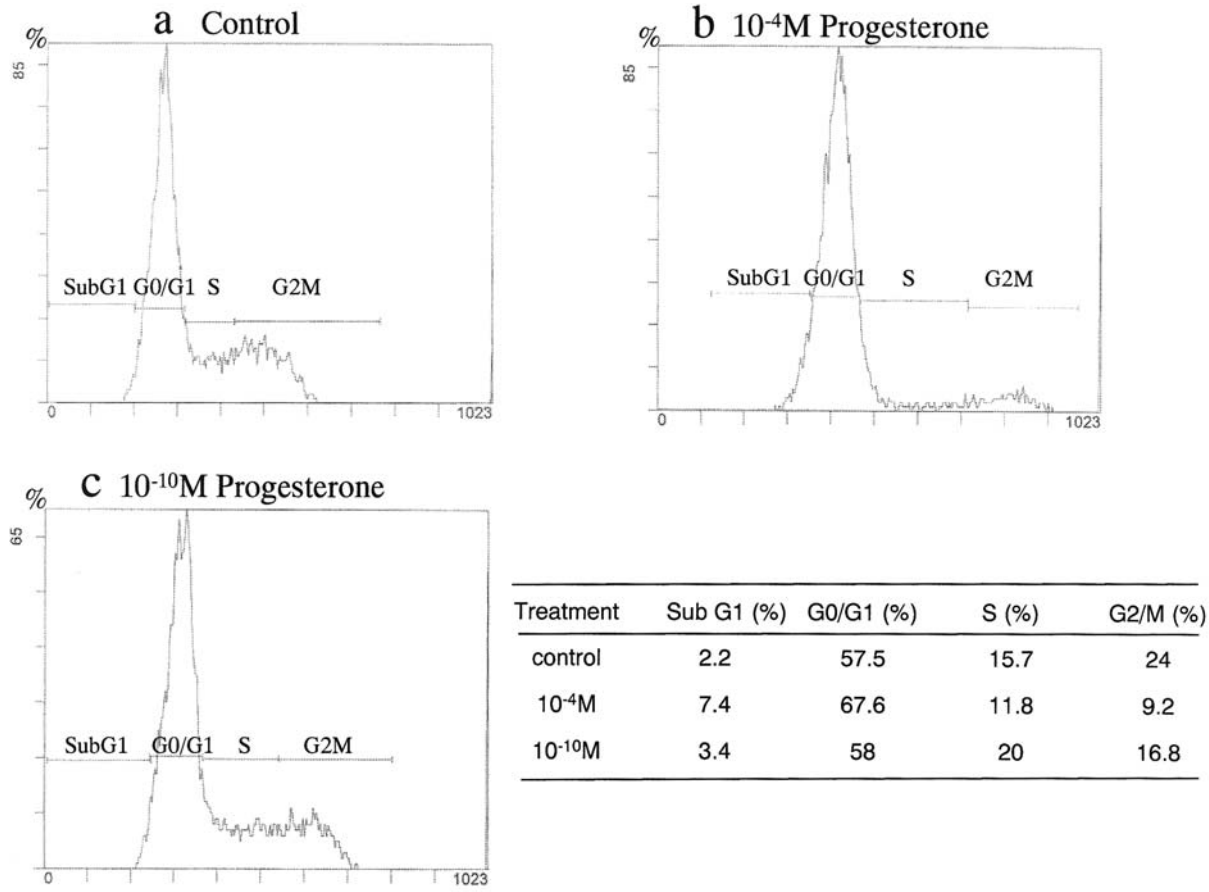
The product of the BRCA1 gene is a 220-kD nuclear phosphoprotein which acts in DNA repair, transcriptional activation, cell cycle regulation and apoptosis (10, 11). Although the potential for altered BRCA1 functions exists in all somatic cells of patients with BRCA1 germ line mutations, the increased risk of cancer in mutations carriers is preferentially observed in hormone-sensitive organs such as breast and ovary, suggesting a possible interaction between BRCA1 and ovarian hormones. The expression of BRCA1 mRNA is induced by 17β estradiol in breast cancer cell lines expressing estrogen receptors. However, BRCA1 was not demonstrated to be an estrogen-responsive gene (12). Nevertheless, it is established that both estrogen and progesterone play a role in the control of normal and tumoral breast proliferation (13, 14). Furthermore, the recent report from the Million Women Study suggests that estrogen-progestagen combinations may increase the risk of breast cancer compared to estrogen only (15). Since data about BRCA1 and progesterone are scarce, we performed a study to investigate whether BRCA1 was regulated in response to progesterone in the MCF7 breast cancer cell line that expresses both estrogen and progesterone receptors (16, 17).

Materials and Methods

Cell line and progesterone treatment. The MCF7 breast cancer cell line was obtained from the ATCC (American Type Culture Collection, Rockville, MD, USA) and cultured in DMEM supplemented with 10% heat-inactivated fetal calf serum, penicillin-streptomycin (100 units/ml) and gentamycin (10 μg/ml) with 5% CO_2 at 37˚C. The culture medium was not depleted in steroids.

Cells were incubated with progesterone (Sigma, St Louis, MO, USA) for 24 or 48 hours at a concentration of 10^{-4} or 10^{-10}M. Controls were cultured in medium without hormone. All experiments were performed in triplicate.

Proliferation analysis. $5x10^5$ cells were plated in 60-mm diameter petri dishes and incubated with progesterone. Methyl-^{3}H thymidine (Amersham, Piscataway, NJ, USA), 1.5μCi/ml, was added 8 hours before the end of incubation. The cells were washed in phosphate-



Treatment	Sub G1 (%)	G0/G1 (%)	S (%)	G2/M (%)
control	2.2	57.5	15.7	24
10^{-4}M	7.4	67.6	11.8	9.2
10^{-10}M	3.4	58	20	16.8

Figure 1. *Flow cytometry of control MCF7 cells (a) and after 48-hour incubation with 10^{-4} M (b) and 10^{-10} M (c) progesterone. Percentage of cells in the different cell cycle phases and in the sub-G1 are given in the table.*

buffered saline (PBS). Cell pellets were resuspended in PBS (NaH_2PO_4 50mM, pH 7.4, NaCl 2M, EDTA 2 mM), sonicated for 10 seconds at 4˚C, and DNA was precipitated with 10% trichloracetic acid. The pellets were dissolved in soluene (Packard Bioscience, Meriden, CT, USA) and radioactivity was measured in a liquid scintillation counter (LKB, Turku, Finland). The DNA concentration of each sample was quantified according to Labarca and Paigen (18).

Ribonuclease protection assay (RPA). mRNAs were quantified with complementary RNA (cRNA) probes and the ribonuclease protection assay was performed on solubilized cells, as previously reported (19). The ^{32}P-labelled antisense RNA probes were generated using T7 RNA polymerase (Promega, Madison, WI, USA) from linearized pBlue-script II SK+ cloning vector (Stratagene, La Jolla, CA, USA), containing the cDNA fragment of human BRCA1 from nucleotide 5192 to 5446 (accession number : U14680) or human cyclin A from nucleotide 244 to 448 (accession number: X51688). cDNA were obtained by PCR and cloned into XhoI/HindIII linearized pBlue-script II SK+. All plasmids were controlled by sequencing. Cohybridization was performed with a human S6 cRNA probe from nucleotide 553 to 683 (accession number : NM_001010) as an internal standard for quantification. RPA and polyacrylamide gel electrophoresis were performed as previously described (19). The gels were quantitatively analysed with the InstantImager (Packard, Groningen, The Netherlands). The ratio of the signals from protected fragments (intracellular BRCA1/S6 mRNA) was calculated to normalize results.

Western blot. Nuclear proteins were prepared as previously described (20), separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membrane. The membranes were incubated with the BRCA1 mouse Ab1 (clone MS110) monoclonal antibody (Oncogene Research, San Diego, CA, USA) and revealed with (HRP)-conjugated goat anti-mouse (Amersham). Membranes were revealed using the ECL system (Amersham).

Cell cycle analysis. The cells were harvested using a rubber policeman, centrifuged and washed in PBS and fixed for 5 minutes in 70% ethanol. After incubation for 30 minutes at room temperature with 160 μg/ml of Rnase A, the cells were stained with 10μg/ml propidium iodide in PBS for 15 minutes in the dark. Analysis of the cell cycle was assessed with an Epics Elite ESP Coulter (Hialeh, FL, USA), with 488 nm excitation and 543 nm emission. Control cells were compared to progesterone incubated cells.

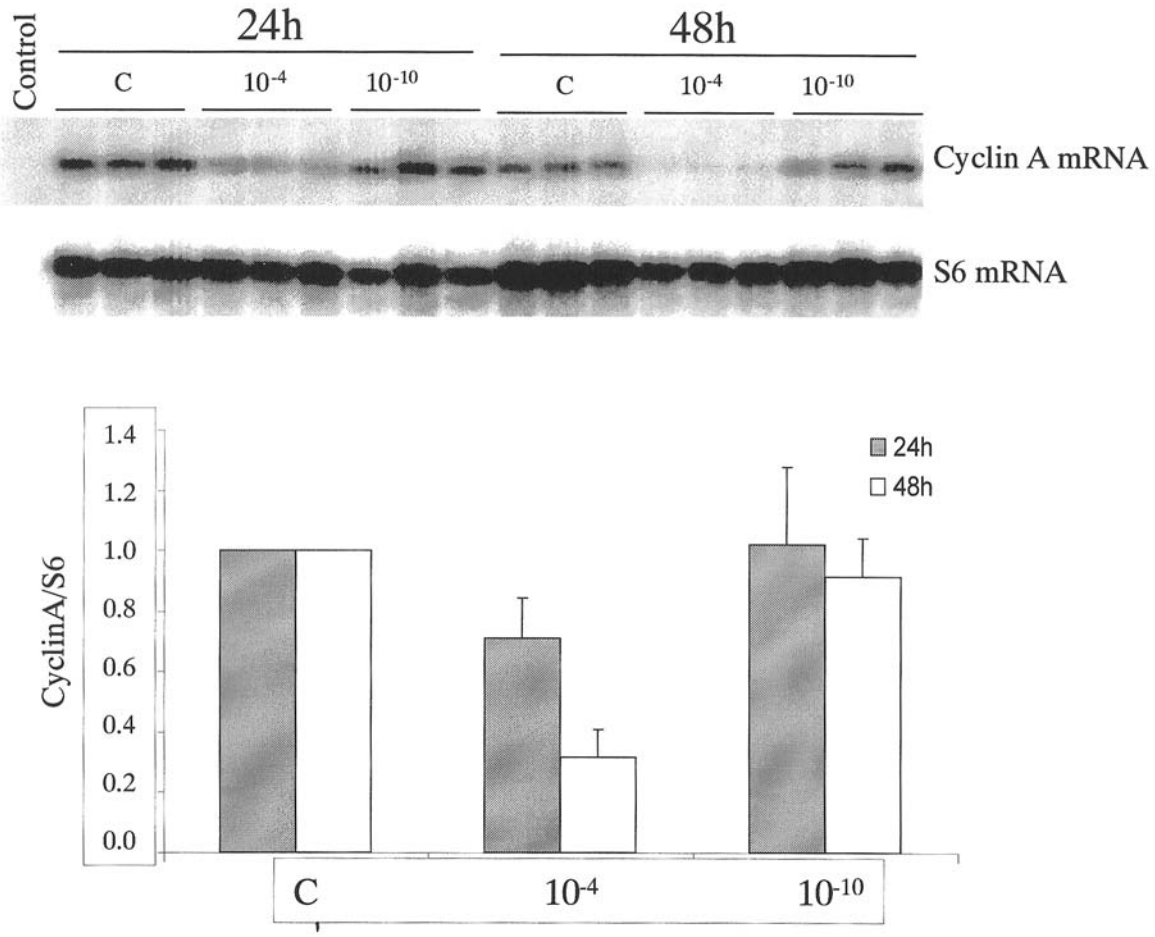


Figure 2. *CyclinA mRNA expression in MCF7 cells evaluated by ribonuclease protection assay after incubation with 10^{-4} M and 10^{-10} M progesterone for 24 and 48 hours. Each bar represents the mean of three independent determinations, corrected for the amount of RNA loading (cyclin A/S6 ratio). The error bar represents the standard deviation.*

Apoptosis analysis. Poly(ADP-ribose) polymerase (PARP) cleavage was analysed on nuclear proteins prepared as described above, by Western blot using an anti-PARP monoclonal antibody (Pharmingen, San Diego, CA, USA) and by flow cytometry.

Results

Proliferation analysis. Treatment with 10^{-4} M progesterone resulted in a 58% reduction of methyl-^{3}H thymidine incorporation after 48 hours (mean 196 dpm/µg DNA, range: 119-287) compared to controls (mean: 443 dpm/µg DNA, range: 267-640), whereas 10^{-10} M progesterone did not modify the methyl-^{3}H thymidine incorporation (mean 462 dpm/µg DNA, range: 201-724).

Cell cycle analysis. Flow cytometry showed at 48 hours that 10^{-4} M progesterone resulted in an increase of MCF7 cells in G0/G1- and a decreased percentage of cells in S- and G2/M-phases (Figure 1b). The percentage of cells in the sub-G1 peak, as a marker of apoptosis, increased from 2.2% to 7.4%. The concentration of 10^{-10} M progesterone did not modify the cell cycle, nor the sub G1 peak (Figure 1c).

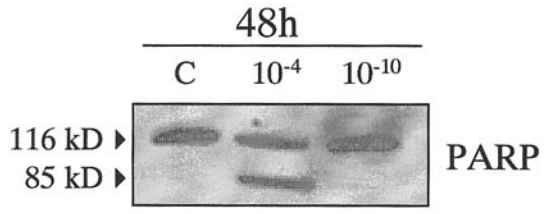


Figure 3. *Western blot of the PARP after incubation with 10^{-4} and 10^{-10} M progesterone for 24 and 48 hours. A cleavage of the 85kD band is visible with 10^{-4} M progesterone.*

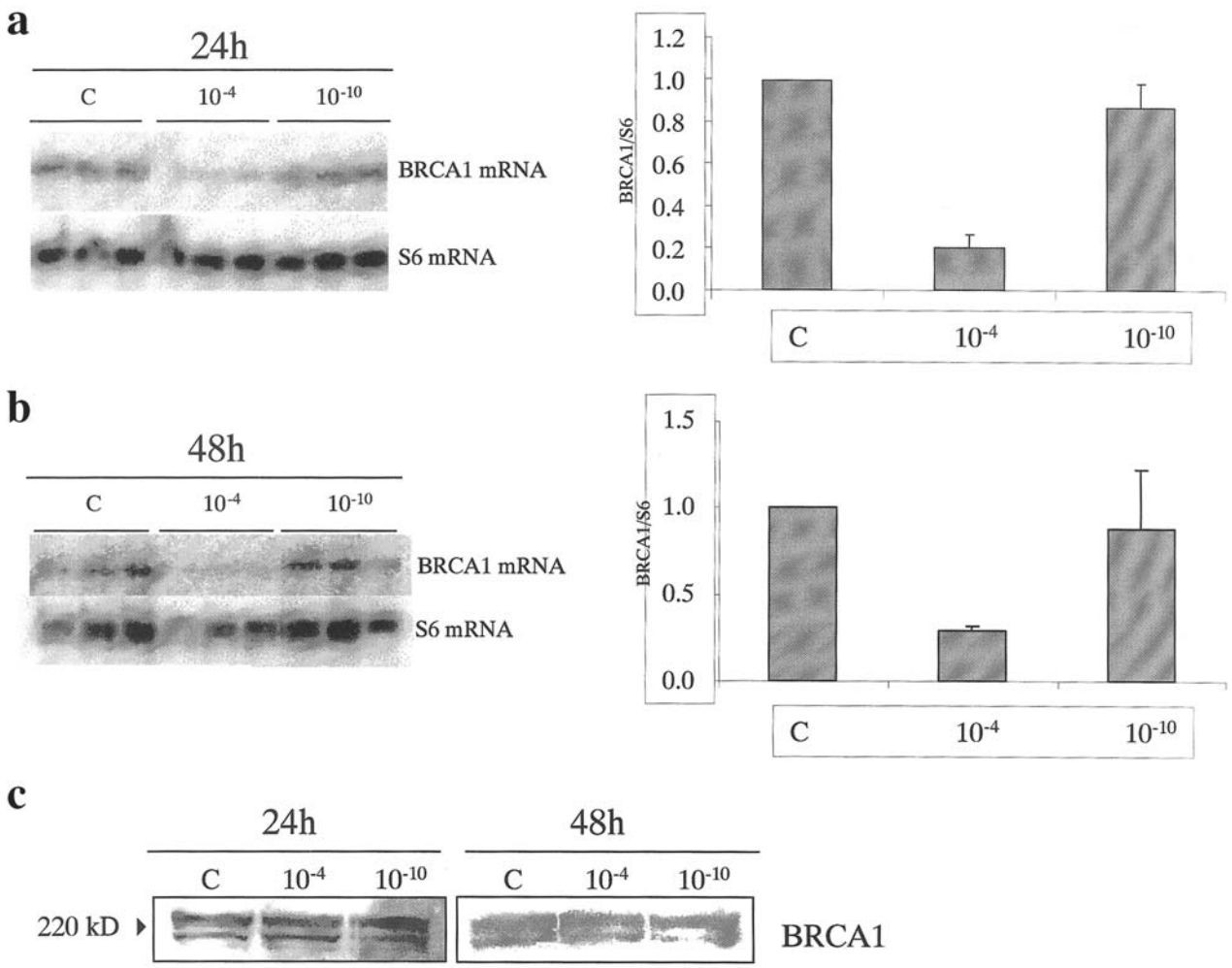


Figure 4. *a and b) BRCA1 mRNA expression in MCF7 cells evaluated by ribonuclease protection assay after incubation with 10^{-4} M and 10^{-10} M progesterone for 24 and 48 hours. Each bar represents the mean of three independent determinations, corrected for the amount of RNA loading (cyclin A/S6 ratio). The error bar represent the standard deviation. c) BRCA1 protein expression in MCF7 cells after incubation with 10^{-4} M and 10^{-10} M progesterone evaluated by Western blot.*

Cyclin AmRNA. Cyclin A mRNA decreased after 24 and 48 hours after incubation with 10^{-4} M progesterone, whereas 10^{-10} M progesterone had no effect (Figure 2).

Apoptosis. In addition to the flow cytometry results, incubation of the MCF7 cells with 10^{-4} M progesterone showed that the hormone induced apoptosis, as demonstrated by the PARP cleavage which presented a 85 KD band. No cleavage of the enzyme was observed with 10^{-10} M progesterone (Figure 3).

BRCA1 mRNA and protein. BRCA1 mRNA decreased strongly 24 and 48 hours after treatment with 10^{-4} M progesterone, whereas 10^{-10} M progesterone had no effect (Figures 4a and 4b). No variation of the BRCA1 protein was evidenced by the Western blot, either at 10^{-4} or 10^{-10} M progesterone (Figure 4c).

Discussion

In this study, we observed in the MCF7 breast cancer cell line that 10^{-4} M progesterone altered the cell cycle, induced apoptosis and down-regulated BRCA1 at least at the mRNA level. The decrease of BRCA1 mRNA followed that of cyclin A mRNA and was therefore attributable to the decreased number of cells entering the cell cycle rather than a direct effect of progesterone on BRCA1. No significant variation in BRCA1 expression was observed at low progesterone concentration (10^{-10} M).

Repression of BRCA1 in breast cancer apoptotic cells was exemplified by treatments with DNA-damaging agents (21).The down-regulation of BRCA1 could be crucial for

cell survival, because it was demonstrated that transfection of BRCA1 in cells with endogenous functional BRCA1 conferred increased sensitivity of the cells to spindle poisons (22). However, the down-regulation of BRCA1 in apoptotic cells could also be explained by the cell cycle modifications since BRCA1 synthesis is cell cycle-dependent, with maximal levels of BRCA1 mRNA reached in late G1- and S-phases (23). More precisely, it was demonstrated that BRCA1 mRNA is increased late in G1 before S-phase, then persists at high levels through G2 and declines in early G1, suggesting that BRCA1 may function at the G1-S checkpoint (23, 24). So it would be difficult to distinguish in our study between a direct hormone regulation of BRCA1 and a secondary response coupled to the hormone effect on the cell cycle. Cyclin A is one of the regulatory molecules of the cell division kinases (cyclin dependent kinase 2). It was previously proposed as a gene marker for late G1-S-phase cells because its level and activity are specifically increased just prior to S-phase entry (25-27). In our study, the decrease in BRCA1 mRNA with 10^{-4} M progesterone followed that of cyclin A, indicating that the observed variation of BRCA1 probably resulted from modifications of the cell cycle rather from a direct interaction between the dimerized progesterone receptor (activated form) with putative BRCA1 regulatory elements.

In contrast, 10^{-10} M progesterone did not modify the proliferation of MCF7 cells in our study and had no effect on BRCA1 mRNA expression. Similar results were reported by Spillman and Bowcock (28) in MCF7 cells incubated with 10^{-8} M progesterone, although the experimental model was different because the cells were cultured in steroid-depleted media for five days and switched to media containing progesterone for an additional five days. Since the progesterone receptor expression is estrogen-dependent in MCF7 cells (29, 30), steroid deprivation for several days may have limited the possibility to demonstrate any progesterone effect in the Spillman and Bowcock's study. This is the reason why we used standard culture conditions.

Gudas *et al.* (27) observed, in the progesterone-positive T47D cell line deprived of steroid hormones for six days and subsequently stimulated with 10^{-8} M progesterone, a delayed increase in BRCA1 mRNA expression with, however, no change in the BRCA1 protein. The steroid depletion protocol used for six days resulted in a significant decrease in the growth of the cells. As the T47D cell is dependent on progesterone for its growth (31), the refeeding with progesterone resulted in an increase of proliferation and BRCA1 mRNA expression that correlated well with an increase of cyclin A mRNA levels suggesting, as in our study, that progesterone affected BRCA1 expression indirectly by modification of the proliferative status.

In our work, no change in BRCA1 protein level accompanied the mRNA decrease observed with 10^{-4} M progesterone. In the absence of BRCA1 half-life evaluation, this observation could not be taken as an evidence against a direct progesterone effect on the BRCA1 gene. Uncoupling of BRCA1 mRNA and protein was also previously reported in other breast cancer cell lines suggesting the possibility of a BRCA1 regulation at the protein level. However, the definitive explanation for this uncoupling remains unclear (13, 27).

In conclusion, our results do not support a direct regulation of BRCA1 by progesterone. Further studies should address whether synthetic progestins regulate BRCA1 in a different manner.

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