Jpn. J. Cancer Res. **91**, 324–330, March 2000

Increased Expression after X-Irradiation of MUC1 in Cultured Human Colon Carcinoma HT-29 Cells

Yun Kang,[1] Kazuya Hirano,[1] Norio Suzuki,[1,3] Atsushi Enomoto,[1] Akinori Morita,[1] Tatsuro Irimura[2] and Kazuo Sakai[1]

[1]Department of Radiation Oncology, Graduate School of Medicine and [2]Laboratory of Cancer Biology and Molecular Immunology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033

The effect of X-irradiation on production of MUC1 was studied with human colon carcinoma HT-29 cells. As evaluated by immunocytochemical staining, the percentages of MUC1-positive cells in cells at 4 days after 6 Gy irradiation and in unirradiated control cells were 52±3.5% (*n*=6) and 26±2.8% (*n*=6), respectively. Flow-cytometric analysis of living cells showed that MUC1 began to rise from day 1, reaching a plateau by day 4 after 6 Gy irradiation. Western blot analysis with monoclonal antibody MY.1E12 against glycosylated MUC1 (mature form) showed dose-dependent increases of two bands (500 and 390 kDa) corresponding to two polymorphic MUC1 alleles. Premature forms of MUC1 (350 and 240 kDa) were detectable with monoclonal antibody HMFG-2 only in irradiated cells, suggesting that new core protein synthesis had been induced. The transcriptional activity of the *MUC1* gene was analyzed in terms of transient expression of MUC1-CAT reporter plasmids containing 5′-flanking sequences of the *MUC1* gene fused to the bacterial chloramphenicol acetyltransferase (*CAT*) gene. The results of CAT assay indicate that enhanced expression of MUC1 in irradiated HT-29 cells was due to upregulation of MUC1 transcription, and required the upstream promoter.

Key words: MUC1 — Radiation — Colon carcinoma cell — Expression — Tumor marker

MUC1 is normally expressed in the epithelial cells of mammary glands, salivary glands, pancreatic ducts, lung, kidneys and stomach.[1)] Normal colonic mucosa usually contain very low levels of glycosylated MUC1 (mature form), whereas malignant tumor cells such as colorectal carcinoma cells often express mature MUC1, particularly at the advanced stages and in metastases.[2–4)] They were found aberrantly expressing MUC1 with or without abnormal cell surface distributions and patterns of glycosylation.[1–6)] Such aberrantly expressed MUC1 was considered a possible candidate as a diagnostic or prognostic tumor marker.[2)] Increased MUC1 expression could also provide tumor cells with an enhanced capacity to metastasize because of the anti-adhesive character of MUC1.[2, 3, 7–9)] We have been involved in studies to clarify mechanisms of tumor metastasis and cellular radiation response.[2, 3, 10–12)] In our recent studies, MUC1 in tumor cells was found to be increased by a novel soluble factor present in normal colon tissue culture conditioned medium (NCCM)[13–16)] or by other agents such as 5-FU.[17)] However, the effect of radiation on MUC1 has not been clarified, so we have been investigating if MUC1 expression in tumor cells could be modified by irradiation and be a biomarker for irradiation effect, and also if modified MUC1 expression is relevant to metastatic behavior.

[3] To whom all correspondence should be addressed.
E-mail: houki@m.u.-tokyo.ac.jp

MATERIALS AND METHODS

Cell culture and X-irradiation Human colon carcinoma HT-29 cells were seeded at 5×10^5 cells/25 cm^2 plastic tissue flasks in 5 ml of 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DME/F12, GIBCO, Grand Island, NY) supplemented with 10% calf bovine serum (CBS, HyClone, Logan, UT).[18)] Cells in the flasks were incubated at 37°C in a humidified atmosphere with 5% CO_2. One day later, cells were exposed at room temperature to different single irradiation doses (Gy) supplied by an X-ray apparatus (200 kV, 20 mA, SHT 250-3, Shimadzu Seisakusho Ltd., Kyoto) at a dose rate of 1.4 Gy/min. Control cells were exposed to the same conditions but not irradiated.

Colony formation assay To measure cell survival, X-irradiated HT-29 cells were diluted and plated at a concentration of 10^2–10^5 cells/25 cm^2 dish (5 ml of medium). After incubation for two weeks, cells were stained with 1% crystal violet and colonies (>50 cells) were counted. The survival curve was obtained by plotting the log of the survival fraction versus the radiation dose.

Monoclonal antibodies (mAbs) mAb MY.1E12 (mouse IgG, specific for glycosylated MUC1) was prepared from hybridoma culture supernatant as described previously.[18)] mAb HMFG-2 (mouse IgG, specific for underglycosylated MUC1) was purchased from COSMO BIO Co., Ltd. (Tokyo).[19)]

Immunocytochemistry and flow-cytometric analysis Adherent cells were harvested by trypsinization and collected by centrifugation for 5 min at 500*g*. Cells (5×10^5) were washed 3 times with ice-cold phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) and 0.1% NaN_3, and incubated with mAb MY.1E12 or with control normal mouse serum for 1 h at 4°C. Each sample was washed with PBS, incubated for 30 min with 100 μl of 1:50 diluted fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Organon Teknika Corp., West Chester, PA) at 4°C, and subsequently incubated with propidium iodide (PI, 2.5 μg/ml in PBS) for 5 min to stain dead cells. Immunofluorescence microscopy was performed under an inverted fluorescence microscope (UFX-DX, DM-510 filter; Nikon, Tokyo). The percentage of MUC1 (green fluorescence)-positive cells was calculated by scoring 500 cells; PI (red fluorescence)-stained dead cells were excluded from quantitation. Flow cytometry was carried out with a Cyto Ace 150 (JASCO, Tokyo). MUC1 was quantified as median green fluorescence intensity and corrected for autofluorescence by subtracting the median fluorescence intensity values obtained with normal mouse serum. Gating conditions for MUC1 were determined based on forward scatter and PI fluorescence pattern.

Western blot analysis Cell pellets (2×10^6) were lysed with 200 μl of sample buffer (1% SDS, 3% β-mercaptoethanol, 5% glycerol, 62.5 m*M* Tris-HCl, pH 6.8). Cell lysates were incubated for 5 min at 100°C and centrifuged for 5 min at 12000*g*. Approximately 60 μg of cell lysates was electrophoretically separated on 4% polyacrylamide gels (SDS-PAGE) and separated components were transblotted.[18] After preincubation for 1 h in 1% BSA in tris-buffered saline (TBS, 20 m*M* Tris-HCl, pH 7.6, 0.9% NaCl), the blotted membranes were incubated with mAb MY.1E12 at 4°C overnight, washed with 0.05% (v/v) Tween-20 in TBS (T-TBS), and further incubated for 1 h with 1:500 diluted peroxidase-conjugated goat anti-mouse immunoglobulins (Dako, Carpinteria, CA) at room temperature. Bound antibodies were visualized with a Konica Immunostaining HRP-1000 (Konica, Tokyo). In some cases MUC1 was desialylated *in situ* by treating the blotted membrane with 10% acetic acid for 1 h at 80°C.[13, 18] After repeated washing with T-TBS and preincubation with 1% BSA in TBS, the membrane was incubated with mAb HMFG-2 at 4°C overnight. As an internal control, western blot analysis was performed with mouse anti-human β-actin mAb (Sigma, St Louis, MO). Murine laminin (400 and 200 kDa; Iwakigarasu, Tokyo) was used as a molecular marker.

Transient transfection and chloramphenicol acetyltransferase (CAT) assays Reporter plasmid (P3C) contained the 5′-flanking sequences of the *MUC1* gene ranging from about positions −2800 to +33 fused to the *CAT* gene.[15, 20, 21] Deletion plasmids sh4-CAT (−531 to +33), sh9-CAT (−485 to +33) and *Sac*I-CAT (have no 5′-flanking sequences of the *MUC1* gene) were prepared as described previously.[15] To normalize the efficiency of individual transfections, pRSV-β-Gal containing the *β-galactosidase* gene under the control of the Rous sarcoma virus promoter was used.[15] HT-29 cells (4×10^6 cells/400 μl) were suspended in Saline G (0.13 *M* NaCl, 5.3 m*M* KCl, 1.1 m*M* KH_2PO_4, 1.1 m*M* Na_2HPO_4, 0.49 m*M* $MgCl_2$, 0.9 m*M* $CaCl_2$, 6.1 m*M* glucose, pH 7.1) and transfected with 7.5 μg of MUC1-CAT reporter plasmids and 7.5 μg of pRSV-β-Gal by electroporation (200 V, 960 μF; "Bio-Rad Gene Pulser," Hercules, CA). The cells were kept for 10 min on ice, then incubated in DME/F12 medium containing 10% CBS for 3 h at 37°C. The transfected cells (either unirradiated or exposed to 6 Gy of X-rays) were incubated for a further 0 to 4 days. Cell pellets (2×10^6) were lysed with 100 μl of 0.25 *M* Tris-HCl (pH 7.4) by three cycles of freeze-thawing. The β-galactosidase activity in each aliquot was measured with a FluoReporte lacZ/Galactosidase Quantitation Kit, then the cell lysates were incubated with 15 μl of fluorescent BODIPY FL chloramphenicol substrate (Molecular Probes, Inc., Eugene, OR), and 0.9 m*M* acetyl coenzyme A at 37°C for 6 h. Fluorescent chloramphenicol and its acetylated products were separated by thin-layer chromatography and photographed under 365 nm UV light (ATTO, Tokyo). Integrated fluorescence intensity was quantified by use of the public domain NIH Image Program (written by Wayne Rashand at the US National Institutes of Health). CAT activity was calculated as the ratio of the fluorescence intensity of acetylated chloramphenicol to that of total chloramphenicol. The CAT induction by X-irradiation was calculated as the ratio of CAT activity of irradiated cell lysates to that of unirradiated lysates.

RESULTS

Viability and survival curve Cell viability at 4 days after 6 Gy irradiation was 65±6.7% (n=6) as determined by the dye exclusion test using erythrosin B (final 0.025% in serum-containing medium). Fig. 1 shows the survival curve determined by colony formation assay of HT-29 cells after X-irradiation (D_0=1.56 Gy; D_q=2.6 Gy).

Increase of MUC1 expression after irradiation HT-29 cells were irradiated with 6 Gy of X-rays and collected from day 0 to day 4 after irradiation. Immunofluorescence microscopy showed that the percentage of MUC1-positive cells was 52±3.5% (n=6) in 6 Gy irradiated cells at day 4, compared with 26±2.8% (n=6) in unirradiated cells (Fig. 2). MUC1 expressed on viable cells was analyzed by flow cytometry. The percentage of MUC1-positive cells (relative fluorescence intensity >10) was increased at day 4 after 6 Gy of X-irradiation (Fig. 3A). In Fig. 3B, MUC1

per cell, quantified and expressed as median fluorescence intensity of living cells, began to rise from day 1, reaching a plateau by day 4 after 6 Gy of X-irradiation (the values at day 5 and day 7 were at similar levels to that of day 4). These results were consistent with the immunocytochemistry analysis (Fig. 2).

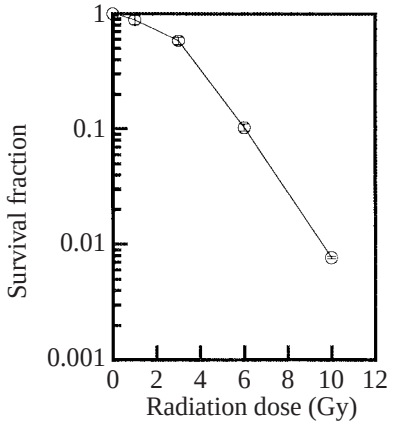

Fig. 1. Survival curve of HT-29 cells after exposure to X-rays. The data represent means±SD of five independent experiments.

Dose-dependent increase of MUC1 and its core proteins after irradiation In order to investigate further the increase of MUC1 after X-irradiation, western blot analyses were performed with mAb MY.1E12 against glycosylated MUC1 and mAb HMFG-2 against underglycosylated MUC1. Fig. 4 shows the electrophoretic profile of MUC1 in HT-29 cells at day 4 after X-irradiation. As is clear from Fig. 4A, MUC1 (500 and 390 kDa) were stainable by mAb MY.1E12 and increased dose-dependently within the examined dose range (1–10 Gy); the 500 kDa bands were weaker than the 390 kDa bands. The mobility of these bands did not change in unirradiated and irradiated cells. MUC1 increased approximately 2- to 3-fold by day 4 after 6 Gy of X-irradiation. Fig. 4B shows MUC1 desialylated *in situ* by treating the blotted membrane with acetic acid and stained with mAb HMFG-2 specific for underglycosylated MUC1. Four bands (500, 390, 350, 240 kDa) were stainable by mAb HMFG-2 and increased dose-dependently. Glycosylated MUC1 500 and 390 kDa bands

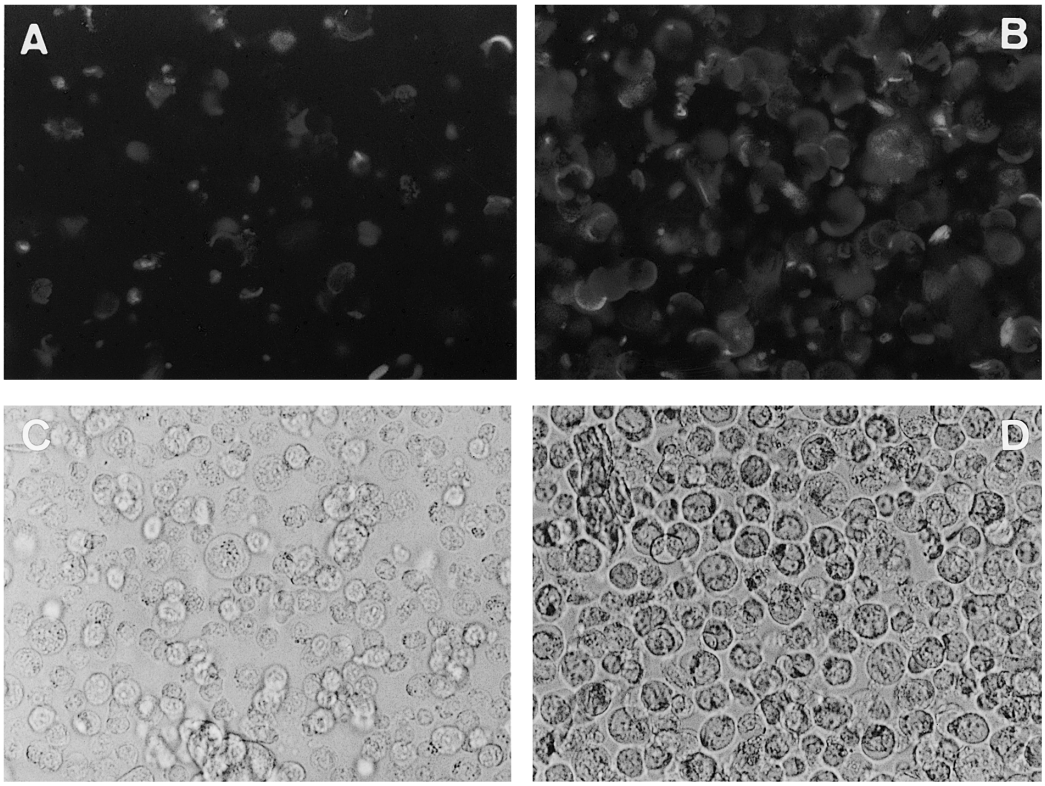

Fig. 2. Immunocytochemistry analysis of MUC1 on HT-29 cells. Unirradiated (A and C) and 6 Gy irradiated cells (B and D) at day 4 were stained with mAb MY.1E12 and FITC-conjugated goat anti-mouse IgG (green). Dead cells were stained with PI (red). The upper (A and B) and lower (C and D) images represent paired fluorescence and phase-contrast micrographs, respectively; ×200.

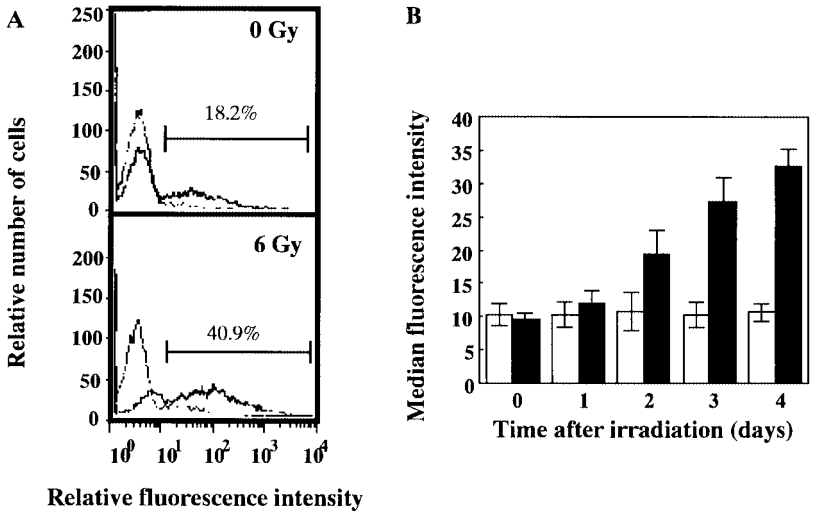

Fig. 3. Time-dependent increase of MUC1 after X-irradiation. Flow-cytometric analysis was performed on HT-29 cells (5×10^3). (A) Unirradiated and 6 Gy irradiated HT-29 cells at day 4 were stained with normal mouse serum (thin line) or mAb MY.1E12 (thick line). (B) Median fluorescence intensity of cells was corrected for autofluorescence. The data represent means±SD of six independent experiments. □ 0 Gy, ■ 6 Gy.

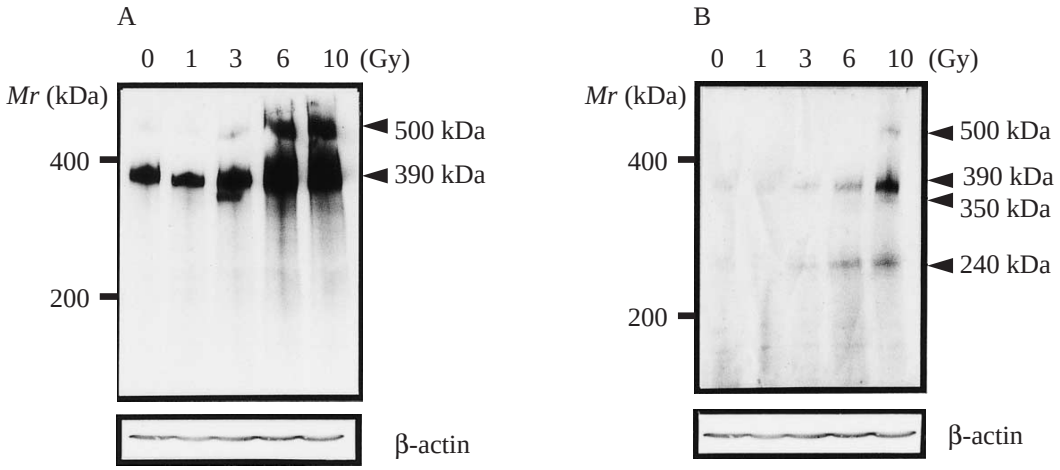

Fig. 4. Dose-dependent increase of MUC1 after X-irradiation. Unirradiated and irradiated HT-29 cells were prepared at day 4 after X-irradiation. (A) Western blot analysis was performed with mAb MY.1E12 against glycosylated MUC1. (B) After blotting onto membranes, MUC1 bands were desialylated by acetic acid *in situ* and stained with mAb HMFG-2 against underglycosylated MUC1. The β-actin blots were used as internal controls. Arrows indicate the approximate position of MUC1.

were not stainable by mAb HMFG-2, unless sialic acids on the termini of carbohydrated side chains were removed (data not shown). MUC1 350 and 240 kDa bands were stainable by mAb HMFG-2 regardless of desialylation treatment (Fig. 4B and data not shown), though the 350 kDa band was weaker than the 240 kDa band. These bands were not detected in unirradiated cells. These 350 and 240 kDa bands probably represent X-ray-induced underglycosylated precursors of MUC1. β-Actin was equally produced in the unirradiated and irradiated cells (Fig. 4).

Upregulation of MUC1 transcription after irradiation

HT-29 cells were transfected with MUC1-CAT reporter plasmids (P3C). In Fig. 5, CAT activities are plotted

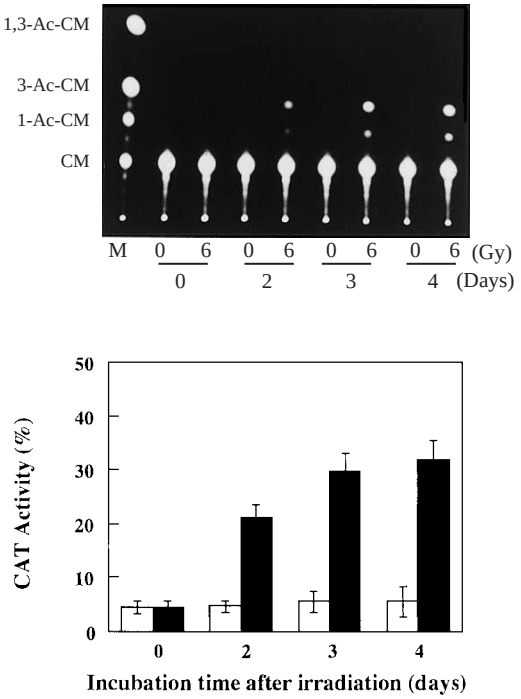

Fig. 5. MUC1 induction after X-irradiation determined by CAT assay. Top: HT-29 cells were transfected with MUC1-CAT reporter plasmids (P3C). The transfected cells (either unirradiated or exposed to 6 Gy of X-rays) were incubated for an additional 0 to 4 days. Fluorescent chloramphenicols and acetylated products are shown at various incubation times. Ac-CM, acetylated chloramphenicol; CM, chloramphenicol; M, marker. Bottom: CAT activity is shown versus incubation time. CAT activity was calculated as percent of the acetylated chloramphenicol with respect to the total chloramphenicol. The data represent means±SD of three independent experiments. □ 0 Gy, ■ 6 Gy.

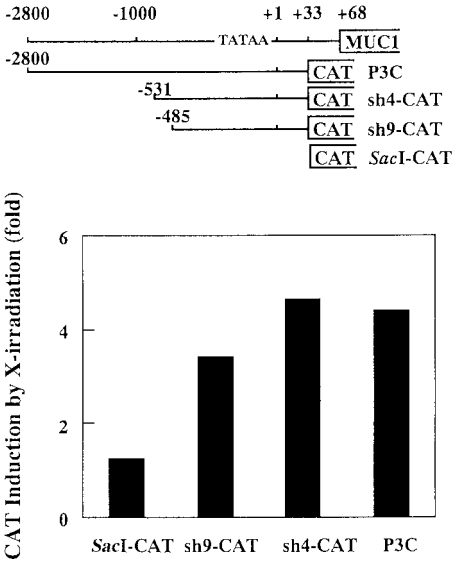

Fig. 6. Deletion experiment of MUC1 promoter. HT-29 cells were transfected with MUC1-CAT reporter plasmids with various constructs (*Sac*I-CAT, sh9-CAT, sh4-CAT or P3C). Numbers indicate the positions of the 5′-flanking sequences of the *MUC1* gene. The CAT induction (fold) was calculated as the ratio of CAT activity of irradiated cell lysate to unirradiated lysate, 3 days after X-irradiation.

against incubation days after 6 Gy irradiation. MUC1 transcription activity in irradiated cells was increased most in the initial 2 days and slightly thereafter, but in unirradiated cells the activity remained at low levels.

In Fig. 6, HT-29 cells were transfected with deletion MUC1-CAT reporter plasmids sh4-CAT, sh9-CAT or *Sac*I-CAT to test whether the upstream promoter was required for X-irradiation-induced MUC1 expression and whether the promoter region responding to X-irradiation overlapped with that for NCCM.[15] The cells were collected after 6 Gy irradiation followed by 3 day incubation. In the cells transfected with *Sac*I-CAT, which contains the *CAT* gene, but had no upstream promoter region of the *MUC1* gene, CAT induction by X-irradiation, expressed as the ratio to unirradiated, was negligible or at unit level, i.e., the same as in unirradiated cells. CAT induction was apparently high in the cells transfected either with sh4-CAT or sh9-CAT, although sh9-CAT was somewhat less effective than sh4-CAT or P3C. Repeated experiments gave similar results.

DISCUSSION

Increase of cellular MUC1 after X-irradiation (1–10 Gy) was demonstrated by immunohistochemistry (Fig. 2), flow cytometry (Fig. 3) and western blotting (Fig. 4A) with mAb MY.1E12. It seems unusual for the effect of X-irradiation to take 3 to 4 days before full induction in a culture system, although the upregulation occurred mostly in the initial 2 days after X-irradiation (Fig. 5). The elongated expression time may be due to maturation and transportation processes with glycosylation in addition to protein synthesis of premature MUC1.[22,23] Another possibility is that *MUC1* gene activation may be a secondary event(s) following activation of other early genes. For example, in the radiation-induced increase of tumor cell collagenase, 48 h is required to upregulate transcription and protein synthesis following early responses of the *Fos* and *Jun* genes.[24–26] Irradiated HT-29 cells might produce unknown autocrine or exocrine soluble factors such as NCCM, which secondarily induce MUC1 production.

Western blot analysis by mAb HMFG-2 showed that desialylated MUC1 (500 and 390 kDa) and precursors of MUC1 (350 and 240 kDa) increased dose-dependently

after X-irradiation (Fig. 4B). These results strongly suggest that the increase of glycosylated 500 and 390 kDa MUC1 seen in irradiated cells was due to the increased synthesis of MUC1 core proteins.

As described in other reports involving Southern blot analyses of MUC1 genomic DNA of various cell lines,[22, 27, 28] HT-29 cells (data not shown) showed two MUC1 alleles with different numbers of tandem repeats. The increased 500 and 390 kDa bands after X-irradiation were considered to be upregulated transcription products of the two MUC1 alleles, and not due to acquisition or increase of additional *MUC1* gene copies.[22]

CAT induction by X-irradiation was seen with sh4-CAT (deleted to −531) and sh9-CAT (deleted to −485), but was negligible with *Sac*I-CAT, lacking the MUC1 promoter region (Fig. 6). These findings indicate that X-irradiation-induced MUC1 expression required the upstream promoter region.

According to our previous study of MUC1 induction by NCCM, the CAT induction was completely abolished by deletion to position −485, and the responsive element was considered to be located between positions −531 and −520.[15] In the present case, the responsive element of X-irradiation-induced MUC1 expression was not the same as that of NCCM. The findings indicate that enhanced expression of MUC1 in irradiated HT-29 cells was due to upregulation of the MUC1 transcription and that the responsive elements are possibly located between positions −485 base pair and the transcriptional start site of the 5′-flanking sequences of the *MUC1* gene. Increased DNA-binding activity after X-irradiation of transcription factors such as NF-kB and Sp-1 has been reported in other target genes such as thymidine kinase and tissue-type plasminogen activator.[29] Kovaric *et al*. also reported that the Sp-1 site at −99 to −90 was necessary for spontaneously expressing MUC1 transcription in breast cancer ZR-75 cells.[21] Deletion plasmid sh9-CAT (−485 to +33) contains the Sp-1 site, although it is not yet clear whether the Sp-1 site is the responsive element in the present radiation-induced MUC1 expression.

The present results are the first to show a time- and dose-dependent increase of MUC1 expression induced by X-irradiation. We further demonstrated that the increase was mediated by upregulation of *MUC1* gene transcription.

ACKNOWLEDGMENTS

This work was supported partly by grants from the Ministry of Education, Science, Sports and Culture, the Ministry of Health and Welfare of Japan, and Ground Research for Space Utilization promoted by NASDA and Japan Space Forum. We thank Dr. M. Yamamoto for valuable comments regarding preparation of MUC1 promoter-CAT constructs and for the generous gift of mAb MY.1E12.

(Received June 29, 1999/Revised September 25, 1999/2nd Revised December 10, 1999/Accepted January 5, 2000)

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