Coordinate Late Expression of Trefoil Peptide Genes (*pS2/TFF1* and *ITF/TFF3*) in Human Breast, Colon, and Gastric Tumor Cells Exposed to X-Rays[1]

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Abstract

The trefoil factors (TFFs) are pleiotropic factors involved in organization and homeostasis of the gastrointestinal tract, estrogen responsiveness, inflammatory disorders, and carcinogenesis. In an earlier study using cDNA array technologies to identify new genes expressed in irradiated cell survivors, we isolated a cDNA clone corresponding to the reported human *TFF1* gene (E. K. Balcer-Kubiczek *et al.,* Int. J. Radiat. Biol., *75*: 529–541, 1999). To determine whether expression of other TFFs is altered by ionizing radiation, we quantified changes in expression of *TFF3* as well as *TFF1* in RNA samples obtained from irradiated and control human tumor breast, colon, and gastric tumor cells and examined expression kinetics up to 2 weeks after irradiation. X-ray-induced *TFF1* and *TFF3* expression profiles were compared with those induced by hydrogen peroxide (H_2O_2) or 17β-estradiol (ES). The results revealed that *TFF1* and *TFF3* mRNA are coinduced by X-irradiation in a subset of the lines, but substantial heterogeneity in their responses was observed in cells derived from a single cell type. *TFF1* and *TFF3* transcriptional response to X-irradiation differed from that to H_2O_2 or ES in the timing of their induction as well as tissue-type dependence, *i.e.,* their induction pattern after X-irradiation was late and sustained, whereas their induction by H_2O_2 or ES was early and transient. *TFF1* mRNA, protein production in the cytoplasm, and secretion in the culture supernatant were coordinately regulated after X-irradiation. There was no requirement for TP53 in this induction. These results demonstrate the existence of a novel class of radiation-responsive genes that might be involved in bystander effects.

Introduction

The TFF[3] genes encode stable M_r 6,000–10,000 proteins that contain a common amino acid sequence reminiscent of the trefoil leaf (Ref. 1 and references therein). Three TFF genes have been identified in humans: estrogen-induced breast cancer-associated peptide (*TFF1*; previously termed *pS2, BCEI, D21S21, pNR-2,* and *HPS2*; Ref. 2), spasmolytic protein 1 (*TFF2*; previously termed *SP* and *SML1*; Ref. 3), and intestinal trefoil factor (*TFF3*; previously termed *ITF*; Ref. 4). These genes are highly expressed in regional-specific but complementary patterns throughout the GI tract (4–9). Normal functions attributed to TFF genes include commitment to differentiation during embryonic development of the GI tract (6), as well as protection against mucosal injury, stabilization of the mucous layer, and acceleration of repair of mucosal damage in the adult GI (10–18). In addition to their role in the homeostasis and repair of GI mucosa, all three TFFs may have a role in tumor biology. Support for this function has come mainly from observations that TFF levels are different in malignant tissues, compared with the corresponding normal tissues (19–31). Two general patterns have emerged from these clinical studies; TFFs tend to be overexpressed in tumors in which normal counterparts express no or low levels of TFFs (*e.g.,* breast) and are absent or reduced in tumors in which normal counterparts express high levels of TFFs (*e.g.,* stomach).

The most completely characterized trefoil gene, *TFF1,* was first isolated from the estrogen receptor-positive MCF7 breast carcinoma cell line (2). In estrogen-treated MCF7 cells, *TFF1* expression is directly controlled at the transcriptional level (2, 32–38) via the estrogen-responsive element in the *TFF1* promoter (34). In other normal or tumor cells, estrogen receptor-independent mechanisms involved in the control of *TFF1* expression may include DNA methylation changes at CpG sites within the promoter/enhancer region (36) or chromatin remodeling (37). Intracellular TFF1 protein has been detected in the cytoplasm (9, 17–19, 33, 36, 38) or the perinuclear space (26, 33). Extracellular TFF1 has been detected in the culture medium (33) or human body fluids (39–43) as a secreted peptide. The induction of *TFF1* mRNA and/or its translation product is a widely used indicator of estrogen receptor function or estrogenic activity of natural, environmental, and manmade chemicals, including anticancer drugs (*e.g.,* tamoxifen), metals (*e.g.,* arsenites), synthetic estrogens (*e.g.,* estradiol and testosterone), plant-derived estrogen analogues (phytoestrogens; *e.g.,* dietary isofla-

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[3] The abbreviations used are: TFF, trefoil factor; GI, gastrointestinal; FBS, fetal bovine serum; ES, 17β-estradiol; DPBS, Dulbecco's PBS; ROS, reactive oxygen species.

vones, genistein, and zearalenol), organochlorides (*e.g.,* polychlorinated biphenyls and pesticides), and phenols (29, 35, 37, 43–53). Except for a previous report from our laboratory (54), there are no reports of nonestrogenic agents that induce TFF1 expression in MCF7 and other human cells.

As described previously (54), by screening 1000 cDNAs randomly selected from an MCF7 cell cDNA library with total cDNA probes prepared from iron ion-irradiated (2.5 Gy) or control MCF7 cells, we isolated and sequenced a cDNA clone corresponding to *TFF1* (GenBank Data Library Accession No. X00474). *TFF1* mRNA showed high-level induction at 7 days but no change in expression at 3 h, compared with matched control samples; this pattern of induction was also observed in 3-h or 7-day RNA samples from MCF7 cells irradiated with fission neutrons (1.2 Gy) or X-rays (5 Gy) and in RNA samples from the stomach carcinoma KATOIII and Hs746T cell lines or the prostate carcinoma PC3 cell line irradiated with X-rays (5 Gy). In the same series of experiments, no induction of *TFF1* mRNA was observed in a MCF7-variant MCF7 ADR^R, the colon carcinoma HT15 cell line and the kidney carcinoma ACHN cell line. We also reported (54) that induction of *TFF1* mRNA by ionizing radiations of different quality was independent of a functional TP53 pathway.

Human TFF genes form a cluster on human chromosome 21q22.3 with all three TFF genes occupying a single 55-kb genomic fragment (55).[4] In our present study, we hypothesized that this proximity may facilitate their coordinate expression in irradiated cells. The combinational use of TFFs by cells has been demonstrated in response to GI injury (1, 10–12, 16), in *TFF1*- or *TFF3*-knockout mice (14, 17), and in normal or malignant tissues (1, 5, 6, 8–12, 20, 24–26, 28, 31). A further point of interest in our present study was the question of which, if any, factor(s) in our model system may affect induction of TFF mRNAs by X-irradiation. Studies of *TFF1* transcription and/or translation in the MCF7 cell line (31–34, 38) showed that potential stimulating factors include estrogen, phenol red, and other organic chemicals in the serum-supplemented growth medium used in our previous experiments (54). Production of oxidative stress subsequent to DNA damage, indicated by increased levels of 7-hydro-8-oxo-2′-deoxyguanosine in the progeny of human cells irradiated with X-rays (1 and 3 Gy), and fission neutrons (0.4 or 1 Gy) have been reported (56). The persistent and enhanced free radical activity reported by Clutton *et al.* (56) suggests one possible epigenetic mechanism for previously observed persistent and delayed *TFF1* mRNA induction by ionizing radiation (54). Therefore, we wanted to know whether and to what extent oxidative stress is involved. In an effort to better understand TFF transcriptional responses to X-irradiation, we sought to: (*a*) compare mRNA expression of *TFF1* and *TFF3* in normal stomach, colon, breast, and several other tissues; (*b*) characterize the radiation-induced expression of *TFF1* and *TFF3* in tumor cells of the human stomach, colon, and breast; and (*c*) determine whether reactive oxygen species, estrogen, or estrogen-like phenol red plays a role in their inducibility.

[4] Website for chromosome 21 map information: http://www.ncbi.nlm.nih.gov/genome/guide.

Materials and Methods

Cell Lines. The human breast carcinoma cell line MCF7 and the Adriamycin-resistant variant MCF7 ADR^R were described previously (57). The stable H_2O_2-resistant variant of the breast epithelial cell line MCF10A, MCF10A/ROS, has been derived and characterized by Dr. P. Gutierrez (Department of Biochemistry and Molecular Biology, and Greenebaum Cancer Center, Baltimore, MD). The resistance of MCF 10A/ROS to H_2O_2 has been associated with a significant, up to 4-fold, elevation of the 7-hydro-8-oxo-2′-deoxyguanosine level, compared with MCF7 or MCF10A (58). The colorectal carcinoma cell line N6CHR3 is an hMLH1-competent variant of the HCT116 cell line (59). The colorectal carcinoma cell lines p53KO and p21KO are, respectively, TP53-null and CDKN1A-null variants of the HCT116 cell line (60, 61). In addition to HCT116 (59–61), the cell lines with a wild-type TP53 phenotype were MCF7 (54, 57, 62–64), MCF10A (65), p21KO (61), LoVo (66), and LS180 (67). The p53KO, p21KO, and N6CHR3 cell lines were a generous gift from the laboratory of Dr. B. Vogelstein (Johns Hopkins Oncology Center, Baltimore, MD). The remaining human carcinoma-derived cell lines in Table 1 were obtained from the American Type Tissue Collection (Manassas, VA). Of cell lines listed in Table 1, only MCF7 cells express a functional estrogen receptor (32–34, 54, 62–65), and only MCF10A cells are negative for growth in nude mice (62–65).

All cells were cultured under conditions recommended by their respective depositors or as described in previous reports (54, 57–59). The routine culture medium contained 5 μg of phenol red/ml and 1 pg of ES. Culture media were 90% IMEM (zinc-option) + 10% FBS for MCF7, 90% RPMI + 10% FBS +10 μg of Adriamycin/ml for MCF7 ADR^R, 95% F12/DMEM + 5% horse serum + 0.5 μg of choleratoxin + 20 ng of epidermal growth factor/ml + 0.5 μg of hydrocortisone/ml + 10 μg of insulin/ml for MCF10A, 95% F12K/DMEM + 5% horse serum + 0.5 μg of choleratoxin + 20 ng of epidermal growth factor/ml + 0.5 μg of hydrocortisone/ml + 10 μg of insulin/ml + 100 μM H_2O_2 for MCF10A/ROS, 90% DMEM (high glucose, low sodium bicarbonate) + 10% FBS for Hs746T, 80% RPMI + 20% FBS for KATOIII, 90% EMEM + 10% FBS for LS180, 90% F12K + 10% FBS for LoVo, 90% DMEM + 10% FBS for HCT15, and 90% McCoy's 5A + 10% FBS for HCT116, p53KO, p21KO, and N6CHR3. Doubling times of cells maintained in the above media were not different and were measured to be approximately 20–24 h.

***TFF1* and *TFF3* mRNA Expression Analysis.** For gene expression analysis, treatments were as follows. X-irradiation (250 kVp, 15 mA, 0.5 mm Cu^{+2}, 1 mm Al^{+3} filtration) was provided as described previously (54, 57). Except for Hs746T cells irradiated at 0 or 10 Gy, all other cells were irradiated at 0 or 5 Gy. In other treatments indicated in Table 1, cells were incubated with 0 or 10 nM ES (Sigma Chemical Co., St. Louis, MO) in 0.01% ethanol for 24 h or with 0 or 0.5 mM H_2O_2 in medium for 1 h.

Cells were prepared for experimentation as described previously (54). Briefly, the experimental protocol consisted of

Table 1 Heterogeneous mRNA expression of *TFF1* and *TFF3* in breast, stomach, or colon cancer cell lines after X-irradiation, hormonal or chemical treatments (Northern blot analysis)

Tissue and cell line	SF5	Reference	Induction of *TFF1* transcription[a]			Induction of *TFF3* transcription[a]		
			X	ES	H_2O_2	X	ES	H_2O_2
Breast								
MCF7	0.018	This study	+	+	+	+	+	−
MCF7ADR^R	0.048	(54)	−	ND	ND	−	ND	ND
MCF10A	0.035	This study	−	−	−	−	−	−
MCF10A/ROS	0.024	This study	−	−	−	−	−	−
Stomach								
Hs746T	0.128	This study	+	+	+	+	+	−
KATOIII	ND	(54)	+	ND	ND	+	ND	ND
Colon								
Ls180	0.008	This study	+	−	+	+	−	+
LoVo	0.006	This study	−	−	−	−	−	−
HCT15	0.024	(54)	−	−	−	−	−	−
HCT116	0.009	(59)	−	−	−	−	−	−
P53KO	0.015	(59)	−	ND	ND	−	ND	ND
P21KO	0.010	This study	−	ND	ND	−	ND	ND
N6CHR3	0.008	(59)	−	ND	ND	−	ND	ND

[a] +, 2-fold or greater induction by the indicated treatment; −, no induction detected; ND, no available data. X, Hs746T cells exposed to 0 or 10 Gy X rays; all the other cells were exposed to 0 or 5 Gy X rays. mRNA levels were assayed 7 days later. ES, treatment of cells with 0 or 10 nM ES for 24 h. mRNA levels were assayed immediately (<15 min) after hormone treatment. H_2O_2, treatment of cells with 0 or 0.5 mM hydrogen peroxide for 1 h: mRNA levels were assayed 1 day later.

seeding cultures at different densities required to avoid growth to confluency during a desired expression time. All groups were seeded and grown for 2 days in standard medium containing untreated serum and phenol red (ph^+/ES^+) to establish the cells in their flasks. Groups to receive treatments under ph^+/ES^+ conditions were treated on day 2 after seeding and continued to culture after treatment in the fresh ph^+/ES^+ medium. Groups to receive treatments under ES-withdrawn conditions were switched on day 2 after seeding from standard ph^+/ES^+ medium to a phenol red-free medium supplemented with dextran-coated, charcoal-filtered serum (HyClone, Provo, UT; stripped medium; ph^-/ES^-) for an additional 2 days before treatment. Stripped medium was renewed daily before treatment, immediately after treatment, and daily during a desired expression period. For consistency, ph^-/ES^- groups had matching ph^+/ES^+ groups with concurrent medium changes. Similar to previous reports (35, 36) and as described later in this report, ES-withdrawn cells maintained full proliferative capability for up to ~1 week in ph^-/ES^- medium, thus limiting the mRNA expression assay duration to 4 days after X-irradiation.

Experimental and control cultures were lysed for RNA extraction at several time intervals after treatment with TRIzol reagent (Life Technologies, Inc., Grand Island, NY) following the manufacturer's protocol, as described previously (54, 68). The isolated total RNA was dissolved in diethylpyrocarbonate-treated water (Sigma) and stored at −80°C until analyzed by Northern blotting or used as a template for reverse transcriptase, as described (54, 68). Ten to 20 μg of each RNA sample/lane were size fractionated by electrophoresis in 1.2% agarose-formaldehyde gels, transferred to Hybond-XL nylon membranes (Amersham Pharmacia), and subsequently UV light cross-linked in a Stratalinker (Stratagene, La Jolla, CA). Northern blot analysis was carried out by sequential hybridization with *TFF1* or *TFF3* cDNA probes in a PerfectHyb-Plus hybridization solution (Sigma Aldrich; Ref. 68).

For additional characterization of *TFF1* or *TFF3* mRNA expression in multiple normal human tissues, a poly(A)$^+$ mRNA dot-blot normalized nylon array (Human MTE system) was purchased from Clontech (Palo Alto, CA). The membrane was sequentially hybridized with ^{32}P-labeled probes containing a partial coding sequence of the human *TFF1* gene or the human *TFF3* gene following the manufacturer's protocols. A *TFF1* probe was generated as described previously (54). A *TFF3* cDNA probe containing nucleotides 13–383 of TFF3 cDNA sequence was obtained as a PCR product using the 5′ primers: GAA GCG CTT GCC GGG AGC AA (for TFF3/F) and CGG AGC CCA CGG TGG TCA TG (for TFF3/R). The ribosomal cDNA probe for the 1.9-kb 18S rRNA was generated as described previously (68). Each cDNA fragment was converted into a random-primed, ^{32}P-labeled cDNA probe by standard methods (69).

TFF1 Protein Expression Analysis. MCF7 cells were seeded in 75-cm^2 flasks, as described above, and sham- or X-irradiated (5 Gy) in ph^-/ES^- medium (13 ml of medium/flask). The media from experimental and control cultures were collected for TFF1 protein expression analysis 4 days after treatment and clarified by centrifugation. Total protein in each of resulting cell-free supernatants was obtained by boiling in protein loading buffer (54). Total protein concentrations were determined by the Bio-Rad protein assay according to the manufacturer's instructions (Bio-Rad Laboratories, Richmond, CA). Equal amounts of protein (20 μg/sample) were separated by 12% SDS-PAGE. Western blot analysis of TFF1 protein in medium was performed with the pS2/pNR-2 Ab-1 clone pS2.1 antibody against human TFF1 (NeoMarkers, Fremont, CA) using enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham) as described previously (54, 58, 59).

To determine cellular localization of TFF1 protein, MCF7 cells were seeded at 5 × 10^4 cells/chamber slide (Lab-TekII; Nalge Nunc International, Naperville, IL) and treated

in ph^-/ES^- medium (2 ml of medium/chamber). The treated cells were then continued to culture in ph^-/ES^- medium for 4 days. The cell cultures were terminated by washing three times in DPBS, followed by fixation in 10% buffered formalin (Sigma) at room temperature for 3 min (38). The fixed cells were washed in DPBS for 5 min each, followed by permeabilization in 0.15% Triton X-100 in DPBS for 15 min. The cells were then blocked at 37°C for 1 h in blocking buffer (10% FBS in DPBS), followed by incubation with the pS2/pNR-2 Ab-1 clone pS2.1 antibody against human TFF1 (1:50; NeoMarkers) at 37°C for 1 h. The cells then were washed four times at 5 min each with DPBS and incubated with a fluorescein-conjugated goat antimouse antibody (1:500). The cells were washed three times at 5 min each with DPBS, followed by staining with 0.1 μg/ml diamidine phenylindole dihydrochloride in Vectashield solution (Vector Laboratory, Burlingame, CA) and examined under a Nikon Eclipse E600 Fluorescence Microscope.

Clonogenic Survival. Survival assays were carried out to determine isosurvival doses for gene expression experiments or differences in plating efficiencies of cells, as indicated in "Results." Survival was determined by a colony-forming ability assay, as described (54, 57, 59). Briefly, appropriate numbers of cells from each treatment group were plated in three 100-mm culture dishes to obtain 100–200 surviving colonies/dish at the end of the assay. Medium was changed weekly. The cultures were incubated for 18–21 days and then fixed and stained.

DNA Fragmentation Assay. Cell morphology after ES withdrawal was examined under an Olympus CK2 light microscope. To assess DNA fragmentation, 10^7 MCF7 cells (floater and attached cell populations) were harvested, washed with PBS, and lysed in DNAzol reagent (Life Technologies, Inc.). Genomic DNA was extracted and ethanol precipitated, according to the protocol recommended by the manufacturer. The precipitated DNA was suspended in Tris-EDTA (pH 8.0) containing RNase (100 μg/ml) and separated by electrophoresis through a 1.5% agarose gel.

Statistical Analysis. Mean values of survival fractions were fitted to the two-parameter linear-quadratic model, as described (54, 59). To determine the amounts of hybrids formed between the transcript of interest and a probe, autoradiographs were analyzed by densitometry using ChemiImager (Alpha Innotech Corp., San Leandro, CA), and results were quantified on the basis of the absolute absorbance of corresponding bands in experimental and control samples, as described (68). Experiments were performed two or more times, and mean or representative results are shown, as indicated in the figure legends. Required calculations were performed using commercial statistical software (PSI-Plot, version 5; Poly Software International, Salt Lake City, UT, and SigmaPlot for Windows, Jandel Scientific, San Rafael, CA).

Results

Expression of *TFF1* or *TFF3* mRNA in Normal Human Tissues. The expression patterns of the two TFF genes in various human tissues showed distinct distributions (Fig. 1).

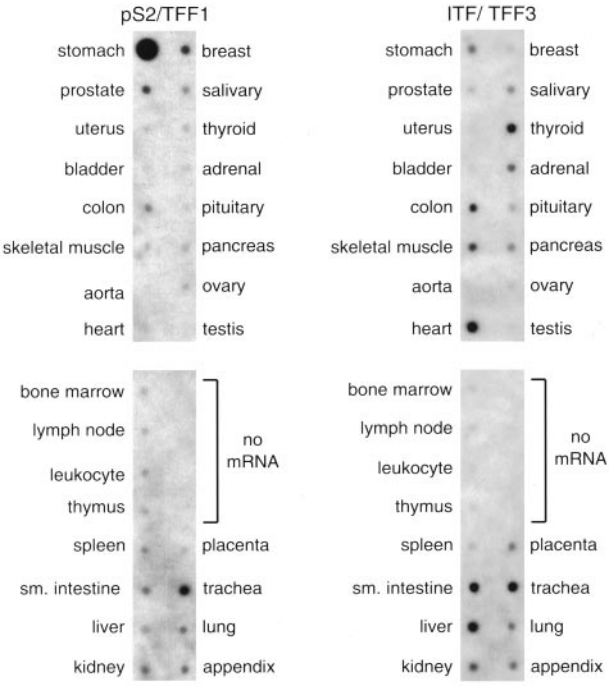

Fig. 1. Expression of trefoil peptide genes, *TFF1* and *TFF3,* in human normal tissues. A multiple tissue expression array of poly(A)$^+$ mRNA was hybridized overnight with randomly primed ^{32}P-labeled *TFF1* or *TFF3* cDNA probes to determine the presence and relative abundance of *TFF1* or *TFF3* transcripts in a broad spectrum of normal tissues. No *TFF1* hybridization signal and weak *TTF3* hybridization signals were detected in several neural tissues, including whole adult and fetal brain (not shown).

We confirmed the maximal level of *TFF1* mRNA in normal stomach as well as high levels of *TFF3* mRNA in normal colon and small intestine reported by others (1, 5, 7). Surprisingly, we observed coexpression of *TFF1* and *TFF3* mRNAs in several normal tissues, including stomach, salivary gland, thyroid, colon, pancreas, small intestine, trachea, liver, lung, kidney, and appendix. The presence of *TFF1* mRNA in normal breast and liver or *TFF3* mRNA in thyroid, skeletal muscle, heart, and trachea has not been reported previously. Minimal or no *TFF1* mRNA was detected in uterus, bladder, aorta, heart, and testis (Fig. 1) and in brain tissues (data not shown). Minimal or no *TFF3* mRNA was detected in uterus, bladder, aorta, and testis (Fig. 1).

Expression of *TFF1* or *TFF3* mRNA in X-irradiated Cells: ph^+/ES^+ Medium Conditions. We demonstrated previously strong time- and dose-dependent variations of *TFF1* mRNA variation in MCF7 cells (54). The cell lines used in this study varied in radiosensitivity according to the data in Table 1 and Fig. 2*A*; 5 Gy was an approximate isosurvival dose in all 13 cell lines (mean surviving fraction of 0.019 $\pm$ 0.004), except for the gastric Hs746T line. On the basis of fitting parameters of full-dose-range Hs746T survival data, the calculated X-ray dose corresponding to 2% survival of Hs746T cells was 10 Gy. Preliminary studies summarized in Table 1 show that under standard estrogenic culture conditions (ph^+/ES^+), cell irradiation at these approximately isosurvival doses resulted in the delayed induction of *TFF1* and

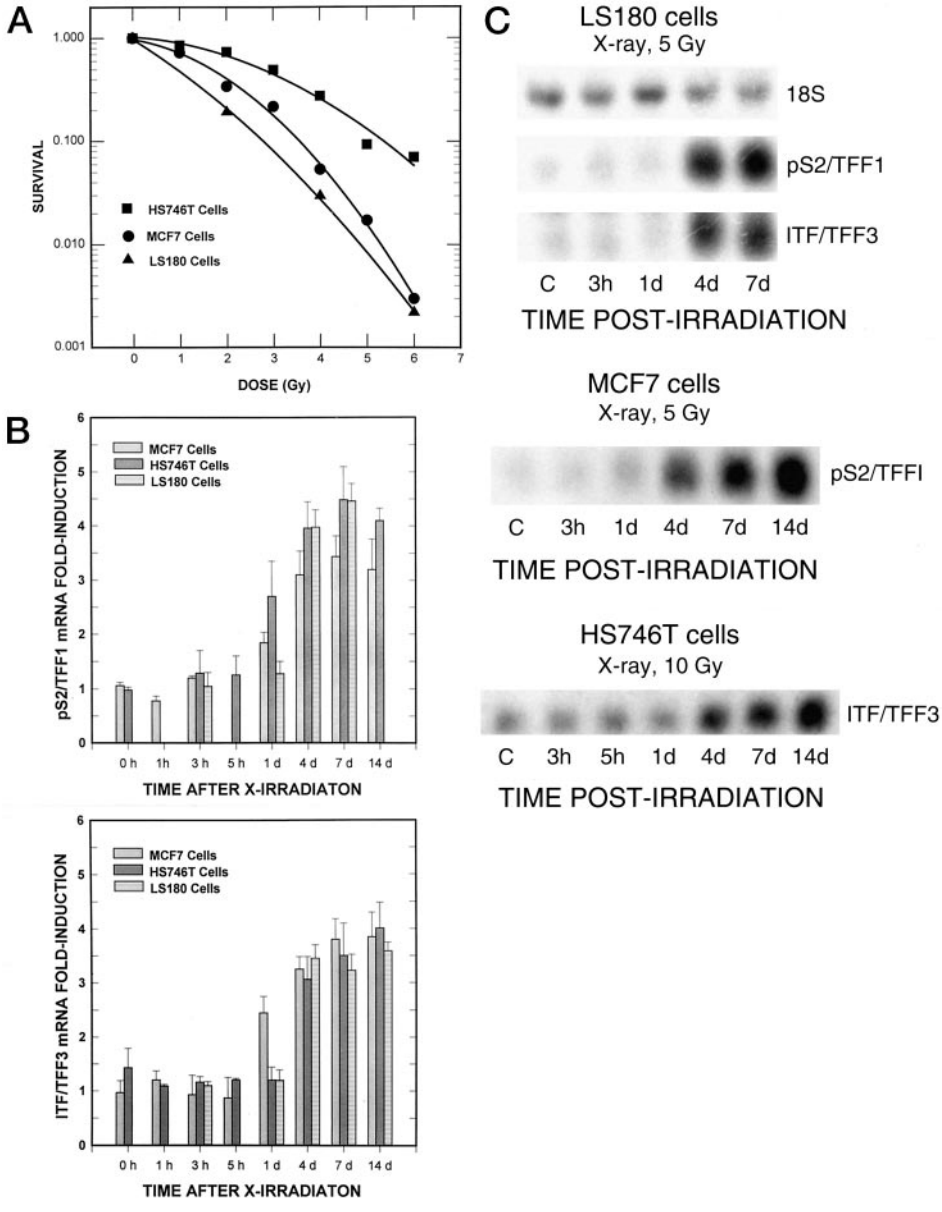

Fig. 2. Effect of X-irradiation on survival and transcriptional activation of trefoil peptide genes, *TFF1* and *TFF3,* in established cell lines from the following human tumor tissues: breast (MCF7), stomach (HS746T), and colon (LS180). *A,* clonogenic survival. *B,* 14-day time course of *TFF1* or *TFF3* mRNA induction under standard estrogenic medium conditions, *i.e.,* cells were cultured in medium containing unmodified serum and phenol red. X-ray doses were 0 or 5 Gy for MCF7 and LS180 cells and 0 or 10 Gy for HS746T cells. Values are expressed relative to mRNA level in contemporaneous controls; *bars,* SE. *C,* examples of Northern analysis of *TFF1* or *TFF3* mRNA in total RNA samples (10–20 μg/lane) isolated from cells that exhibited positive responses to X-irradiation in preliminary screening of cell lines listed in Table 1. Stripped membranes were hybridized with ^{32}P-labeled human probe for the 18S ribosomal gene.

TFF3 mRNAs, relative to earlier by ES, H_2O_2 in 1 of 4 breast lines, 2 of 2 gastric lines, and 1 of 7 colon lines (MCF7, Hs746T and KATOIII, Ls180, respectively). Fig. 2*B* shows the kinetics of the *TFF1* and *TFF3* transcription in three cell lines that have tested positive for the responses to X-rays at the 7-day time point (MCF7, Hs746T, and Ls180; Table 1); examples of Northern blots underlying these data are shown in Fig. 2*C*. Relative to basal levels, *TFF1* and *TFF3* mRNA levels in the MCF7, Hs746T, and Ls180 cell lines increased at least 3-fold at 4 days after X-irradiation and remained at that elevated level through 7–14 days after X-irradiation.

To determine whether the above observations were generalizable with respect to ROS-producing DNA-damaging agents, we treated MCF7, Hs746T, and Ls180 cells with H_2O_2. The shape of survival curves was close to exponential in contrast with those observed with X-irradiation, but interestingly, the order of sensitivity to H_2O_2 was the same as that observed with X-rays, *i.e.,* Hs746T cells were the most resistant and Ls180 cells the most sensitive, whereas MCF7 cells showed an intermediate sensitivity to both agents (Fig. 2*A versus* Fig. 3*A*). In the preliminary studies, we observed strong time-dependent but weak concentration-dependent increases of *TFF1* gene transcription in MCF7 cells after exposure to H_2O_2, and our subsequent studies were done at a single H_2O_2 dose (0.5 mM for 1 h). The data presented in Table 1 and in Fig. 3*B* show that H_2O_2 administration can indeed lead to increased *TFF1* expression in MCF7, Hs746T, and Ls180 cell lines. However, an increase in level of *TFF1* mRNA was only observed at 1 day but not at later time points after H_2O_2 treatment Also, in contrast to the effects of X-irradiation in Fig. 2*B, TFF1* and *TFF3* were generally not coexpressed. In fact, induction of *TFF3* mRNA TFF3 by H_2O_2

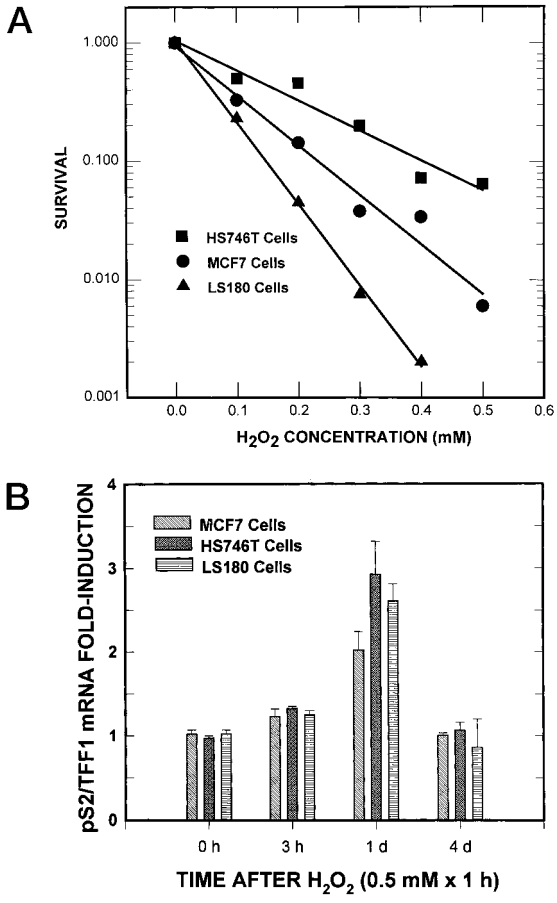

Fig. 3. Effect of hydrogen peroxide on survival and transcriptional activation of *TFF1* in established cell lines from the following human tumor tissues: breast (MCF7), stomach (HS746T), and colon (LS180). *A,* clonogenic survival. *B,* time course of *TFF1* mRNA induction by H_2O_2 (0.5 mM for 1 h) under standard estrogenic medium conditions, *i.e.,* cells were cultured in medium containing unmodified serum and phenol red. Values are expressed relative to mRNA level in contemporaneous controls. Coinduction of *TFF1* and *TFF3* mRNA was detected only in LS180 cells (see Table 1). *Bars,* SE. *C,* example of Northern analysis of *TFF1* mRNA in total RNA samples (10–20 μg/lane) isolated from HS746 cells. A representative sample of ethidium bromide-stained membrane is shown to demonstrate RNA quality and equal loading.

was observed only in the Ls180 cell line, in which *TFF1* mRNA was also induced *TFF3* by H_2O_2 (Table 1). Lack of transcriptional activation of *TFF3* by H_2O_2 in MCF7 and Hs746T cells was confirmed by long exposure of the autoradiographs and, independently, by semiquantitative reverse transcription-PCR (data not shown).

Expression of *TFF1* and *TFF3* mRNA in X-irradiated Cells: ph^+/ES^+ *versus* ph^-/ES^- Medium Conditions. To evaluate the effect of culture medium-associated factors (*e.g.,* the presence of ES in untreated serum) on *TFF1* and *TFF3* mRNA expression patterns induced by X-irradiation, we next examined *TFF1* and *TFF3* mRNAs in two ES-responsive (MCF7 and Hs746T) cell lines (Table 1) after X-irradiation under estrogenic (ph^+/ES^+) and ES-withdrawn (ph^-/ES^-) conditions. X-ray doses were 0 or 5 Gy for MCF7 cells and 0 or 10 Gy for Hs746T cells. As described in "Materials and Methods," maximal time after exposure was 4 days under ph^-/ES^- conditions, thus limiting the mRNA expression assay duration overall. Compared with estrogenic conditions, removal of ES and phenol red completely abolished *TFF1* and *TFF3* mRNA expression in control cells as well as at earlier time points, *i.e.,* earlier than 4 days after irradiation (Fig. 4, *A versus B*; and data not shown). The control results obtained for parallel cultures maintained and treated in estrogenic medium up to 4 days after X-irradiation (Fig. 4*B*; and data not shown) were similar to those in Fig. 2*B*.

Additional studies compared *TFF1* and *TFF3* mRNAs in ES-treated MCF7 and Hs746T cells under assay conditions similar to those in the above experiments with X-rays. Our positive control results (Fig. 4*C*; and data not shown) correlated well with previously reported results of similar experiments (2, 31–35): (*a*) *TFF1* or *TFF3* mRNAs were undetectable in control hormone-withdrawn cells, but there was a detectable transcription level in the absence of any treatment in cells grown in standard media (a representative example in Fig. 4*C, Lane 1 versus Lane 3*); and (*b*) compared with the corresponding basal levels, induction by similar ES treatments (10 nM for 24 h) was at least 5-fold higher under the ph^-/ES^- than under ph^+/ES^+ conditions (Fig. 4*C, Lane 2 versus Lane 4*). Fig. 4*D* shows a 7-day time course of *TFF1* and *TFF3* mRNA induction after ES treatment of estrogen-responsive MCF7 and Hs746T cells under ph^+/ES^+ conditions. There was a similarly marked increase (~7-fold) of *TFF1* mRNA in total RNA isolated from ES-treated MCF7 and Hs746T cells at 0 and 3 h after treatment, as compared to baseline in control cells; TFF1 mRNA returned to baseline within 1 day and remained at that level at 4 and 7 days posttreatment (Fig. 4D, *top*). Figure 4D (*bottom*) shows the relative intensity of the hybridization signal obtained when TFF3 cDNA probe was hybridized to total RNA samples in Fig. 4D (*top*). ES induced maximum amounts of TFF3 mRNA within 24 h of ES treatment (0 h posttreatment) and these were approximately 10-fold more (MCF7 cells) or 5-fold more (Hs746T cells) than untreated cells. The amount of TFF3 mRNA remained elevated at 3 h posttreatment and declined to baseline at later time points (1, 4, and 7 days posttreatment).

We routinely observed that transfer of ES-responsive cells (MCF7 and Hs746T) from ph^+/ES^+ medium into ph^-/ES^- medium led to decreased cell-to-cell adhesion but not cell-to-substratum adhesion after 2 days in ph^-/ES^- medium. To characterize the consequences of ES withdrawal, MCF7 and Hs746T cells were assayed for viability and DNA fragmentation. Plating efficiency or survival of ES-withdrawn cells in the ph^+/ES^+ medium was similar to that of cells grown and assayed under estrogenic conditions. No significant colony formation was observed when MCF7 or Hs746T cells were directly plated into the ph^-/ES^- medium (estimated plating efficiency of less than 0.5%). Apoptosis following ES withdrawal and prevention of apoptosis by ES are well documented for MCF7 cells (63, 64), and these effects have been confirmed in our study. Transfer of MCF7 cells, from ph^+/ES^+ medium into ph^-/ES^- medium induced DNA fragmentation (>400 bp in size) within 2 days of incubation period, which was effectively inhibited by the addition of ES (10 nM

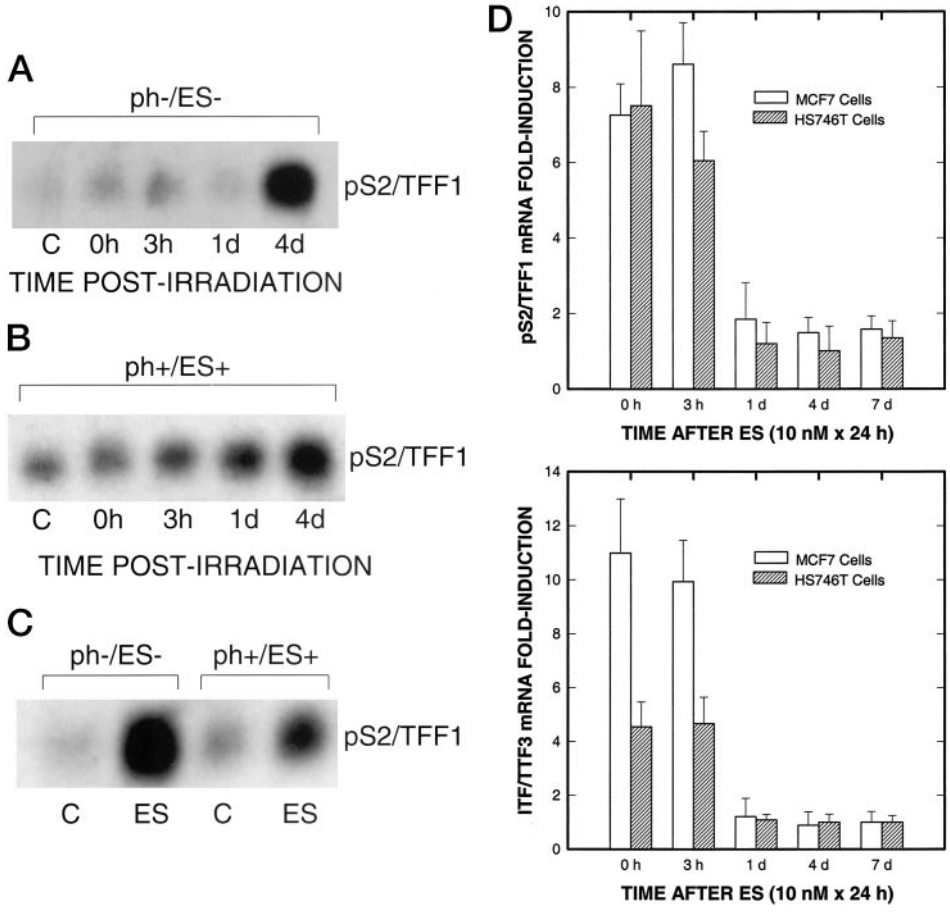

Fig. 4. A and *B,* comparison of time course of *TFF1* mRNA induction under estrogen-withdrawn (denoted ph^-/ES^-) and estrogenic medium (denoted ph^+/ES^+) conditions in X-irradiated MCF7 cells (0 or 5 Gy at time 0 h). Note the reduction of the background associated with phenol red- and serum-dependent mRNA induction in *Lanes C, 0h, 3h,* and *1d*. *C,* comparison of *TFF1* mRNA induction under ph^-/ES^- or ph^+/ES^+ medium conditions in ES-treated MCF7 cells (ES; 0 or 10 nM for 24 h). *D,* 7-day time course of TFF1 and TFF3 mRNA induction under estrogenic medium conditions in estrogen-responsive MCF7 and Hs746T cells treated with 17-β-estradiol (0 or 10 nM for 24 h). Values are expressed relative to mRNA level in untreated cells. *Bars*, SE.

for 24 h) to stripped medium. DNA fragmentation was observed again following re-exposure of cells to ph^-/ES^- medium (>200 bp in size).

Localization of TFF1 Protein in X-irradiated MCF7 Cells: ph^-/ES^- Medium Conditions. To determine whether *TFF1* mRNA made by cells in response to X-irradiation was similarly translated as was *TFF1* mRNA made in response to ES (9, 17–19, 33, 36, 38), we performed immunochemical analysis of MCF7 cells exposed to 5 Gy under ph^-/ES^- conditions and fixed at time points indicated in Fig. 4, *A* and *B*. Fig. 5*A* (*top*) shows that the TFF1 protein was expressed exclusively in the cytoplasm of X-irradiated MCF7 4 days after exposure. No cytoplasmic staining was observed in control samples (Fig. 5*B, bottom*). The timing of induction of TFF1 protein in the cytoplasm therefore coincided closely with an increase in the level of *TFF1* mRNA in X-irradiated MCF7 cells treated and assayed under ES-withdrawn conditions (Fig. 4*A*).

To determine whether the TFF1 protein was topographically restricted to the cytoplasm, ph^-/ES^- medium overlying X-irradiated MCF7 cell monolayers was analyzed by Western blotting for the presence of TFF1 protein. Fig. 5*C* shows that TFF1 production in a sample taken 4 days after 5 Gy of X-rays; no TFF1 protein was detected in the culture supernatant of unirradiated cells (Fig. 5*B, Lane 1*). The secreted protein size of M_r <10,000 detected by Western blot analysis of total protein in the culture supernatant of irradiated MCF7 cells was consistent with the data of Nunez *et al.* (33) and Martin *et al.* (36), who estimated the size of the secreted protein to be M_r 6,500–7,000.

Discussion

We report coordinate regulation of steady-state TFF mRNAs, protein production, and secretion after X-irradiation of human cells. These findings, the first to directly demonstrate protein production with delayed kinetics in irradiated culture medium, are of interest in view of recent observations of radiation-induced epigenetic effects, including radiation-induced genomic instability and/or bystander effects (70–72). TFF1 (and probably TFF3) proteins are candidates for transferable bystander factors because they exhibit several expected characteristics, including stability, small size of M_r 1,000–10,000, dependence on cell density at the time of irradiation, and no wild-type TP53 requirement (Refs. 70–72 and references therein). Moreover, induction of *TFF1* and *TFF3* mRNA, similar to the bystander effect, has been observed in some but not all investigated cell lines, indicating a strong dependence on genetic background of the cells (71). Whether TFF1 and/or TFF3 protein-containing medium elicits a response in nonirradiated cells is an important question that remains to be addressed.

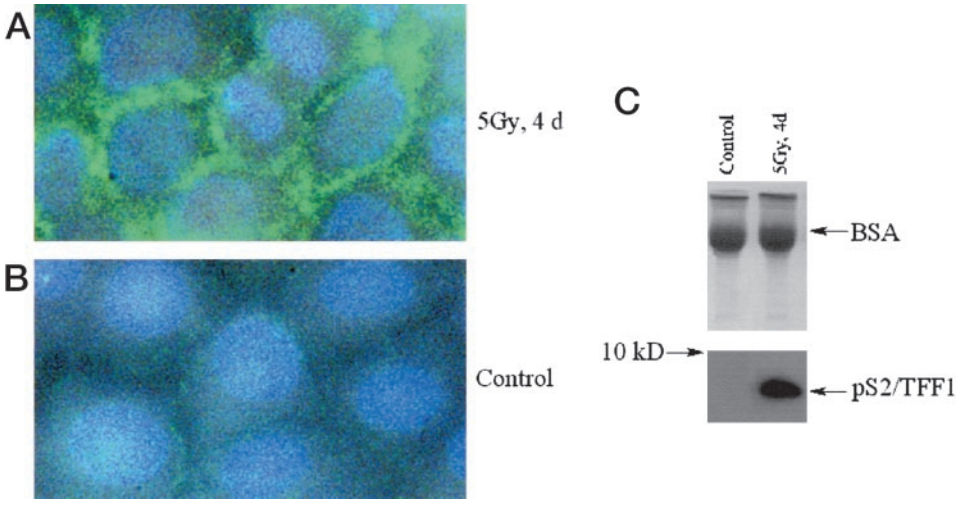

Fig. 5. A, cytoplasmic localization of TFF1 protein in X-irradiated cells. MCF7 cells were cultured for 4 days after 5 Gy in ph^-/ES^- medium, fixed, and subjected to immunohistostaining with anti-TFF1 polyclonal antibody (*green*). Cells were counterstained with diamidine phenylindole dihydrochloride (*blue*). *B,* matched control MCF7 cells. *C,* detection of secreted TFF1 protein in ph^-/ES^- medium 4 days after 5 Gy by Western blot analysis. Coomassie Brilliant Blue-stained gel is shown to demonstrate equal loading of total proteins.

We have described *TFF1* and *TFF3* as genes that exhibit delayed and prolonged expression at the cellular level after exposure to ionizing radiation. These temporal features of expression distinguish *TFF1* and *TFF3* from previously characterized, radiation-responsive genes, the transcript levels of which were reported to be altered typically by 6 h after radiation before returning to basal levels within 24 h (54, 73–78). Using the expression time classification of Yu *et al.* (73), response of these genes thus could be described as "early" and "transient." Products of many of these early genes are known to take part in DNA repair, growth arrest, and apoptosis. Thus, radiation-responsive genes are also often described as stress-associated genes, because transcripts and proteins are induced or suppressed by a variety of agents, including γ- or X-irradiation, chemotherapeutic agents, H_2O_2, UV, hypoxia, and heat. Interestingly, those early-responding genes show a weak dependence on the nature of the induction signal. Examples include TP53-dependent genes, such as *BAX, MCL1, MDM2, GADD45, PIG3, MAT8, DR5,* and *14-3-3*σ, as well as TP53-independent genes, such as *CSA19, FOS, JUNB, MYC, MBP1, TGF*β, and *ATF3* (73–77). In contrast, TFF response after X-irradiation *versus* ES or H_2O_2 was significantly different both in terms of the extent and timing (*e.g.,* Fig. 2*B versus* Fig. 3*B*). On the basis of the above comparison, our present results and those published earlier (54) have identified trefoil factors as a novel class of radiation-responsive genes.

DNA damage induced by H_2O_2 or X-rays is largely based on free OH radicals (79). The observed differences in cell survival and TFF gene expression following H_2O_2 *versus* X-rays may be due to differences in the spatial and temporal distribution of damaging events from these agents. Free radicals produced by amine oxidase in serum may contribute to the stress experienced by cells in culture posttreatment (80), as evidenced by the passage number-dependent increase of transcript levels of damage/stress-associated genes, such as *TP53, MDM2,* and *p16INK4A* (81), as well as telomerase-dependent replicative aging (82). Culture environment-related ROS damage can be excluded as a contributing factor in our study because: (*a*) the *TFF1* or *TFF3* messages at different time points were analyzed in cell populations established from a single stock culture; (*b*) experimental cultures remained at the same passage number after treatment; and (*c*) no coexpression of *TFF1* and *TFF3* after incubation with H_2O_2 was seen in the cell lines in which coexpression of the two mRNAs was observed after X-irradiation. With regard to the medium- and serum-dependent background mRNA induction, the expression data from X-ray and ES studies (Figs. 2 and 4) demonstrate that the presence of phenol red and other estrogens in the culture medium might modify the onset and magnitude of both the basal and induced TFF mRNA levels. However, the major feature of strong induction in X-irradiated cells past 1 day is preserved. Thus, our earlier hypothesis that the culture conditions modify the gene expression profile may be valid.

Similar to the results from other gene profiling studies involving genotoxic agents (73–75), we found marked heterogeneity in TFF transcriptional responses to X-irradiation, ES, and H_2O_2 among cancer cells derived from the same stem cell type. We used three sets of engineered cell lines derived from breast and colon to assess codependence of TFF expression on specific phenotype-associated markers. For example, MCF7 cells and Adriamycin-resistant variant $MCF7/ADR^R$ cells differ in TP53 status in addition to MDR1 status (57, 67), whereas H_2O_2-resistant MCF10A/ROS cells overexpress *BCL2* mRNA, compared with MCF10A cells (58). With regard to the HCT116 cell line and derived variants, alterations secondary to manipulation of TP53 and MLH1 function in, respectively, P53KO and N6CHR3 cells and include overexpression of *MYC, BNIP3,* and *AIP4* (formerly, *survivin*), relative to HCT116 cells (59). We found no correlation between the basal or agent-induced of *TFF1* or *TFF3* mRNA levels and the above markers. The molecular links between normal *versus* disease genetic background and TFF expression also remain enigmatic. Referring to Fig. 1, one or both of these genes are physiologically expressed in normal breast, stomach, and colon, but only in a proportion of cell lines derived from tumors of breast or the GI tract. Thus, additional factors seem to be involved in the control of TFF

transcription in normal tissues as well as breast, gastric, and colon cancer models.

The X-ray results in Fig. 2 are in excellent agreement with our previous results demonstrating the time- and dose-dependent induction of *TFF1* transcription in MCF7 cells (54). Referring next to our results with H_2O_2, the present data on H_2O_2 toxicity in the MCF7 cell line in Fig. 3*A* agree with the published MCF7 cell survival data (82) which were obtained H_2O_2 concentrations similar to those used in our study. The effect of H_2O_2 on Hs746T or Ls180 cell survival has not been examined previously. However, the exponential survival curves for oxidative injury resulting from H_2O_2 could be expected from the association of lethal events with the induction of DNA double-strand breaks by H_2O_2 (79).

Rapid activation of TFF1 transcription in response to ES under estrogenic and estrogen-withdrawn conditions shown in Fig. 4*C* was previously reported by other investigators (32–38), in effect lending validity to the current results with other agents. The pattern of TFF1 and TFF3 mRNA expression after ES (Fig. 4*D*) has not been studied previously. The observation that gastric and breast cells co-express ES-regulated TFF1 and TFF3 mRNA confirms the data of Poulsom *et al*. (20) and May and Westley (31) but is in contrast to that of other investigators (7, 26, 84). We have also confirmed the growth-suppressive and pro-apoptotic effects of ES withdrawal (35, 63, 64). Growth inhibition and apoptosis in cells, following ES withdrawal, coincided with the reduction of TFF1 and TFF3 mRNA levels in our studies. We have also reported that the inhibition of apoptosis by ES may occur in association with transcriptional activation of both TFF1 and TFF3 in human cells. These findings may suggest growth-promoting and anti-apoptotic activity of TFFs. Indeed, evidence supporting this role for TFF1 and TFF3 has been reported by some investigators (23, 35, 51, 52, 84, 85) but there is no consensus as of yet (86).

The focus of this study was on characterization of TFF genes in breast and GI cell lines after X-irradiation and comparison of the induced expression patterns with those induced by other environmental stresses. Malignant tumors at these sites are commonly treated with radiation. Moreover, the normal breast and GI tract, including stomach and colon, are particularly sensitive to the carcinogenic effects of ionizing radiation (87). Additional clinical relevance of *TFF1* and/or *TFF3* might also be established for other normal tissues, specifically salivary gland, heart, trachea, liver, lung, and kidney, because these tissues are unavoidably irradiated in the course of radiotherapy of head and neck, breast, lung, colorectal, and esophageal cancers. Viewed in the context of radiation therapy as well as a health risk assessment from radiation exposure, it is important to learn more about how the expression of TFF genes is remodeled during changes in physiology or environment, to define the sets of genes which are activated by different TFFs, to determine how these sets of activated genes differ as a function of cell type, and finally, to determine the pathogenic importance of persistent TFF alteration in the expression of late radiation-induced tissue damage.

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