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Curcumin Downregulates H19 Gene Transcription in Tumor Cells

Renata Novak Kujundžić,[1]* Ivana Grbeša,[1] Mirko Ivkić,[2] Meena Katdare,[3] and Koraljka Gall-Trošelj[1,3]

[1]Division of Molecular Medicine, Ruđer Bošković Institute, Bijenička 54, 10000 Zagreb, Croatia
[2]Department of Otorhinolaryngology and Head and Neck Surgery, Sestre Milosrdnice University Hospital, 10000 Zagreb, Croatia
[3]Department of Surgery, Weill Medical College, Cornell University, New York, New York

Abstract Curcumin (diferuloymethane), a natural compound used in traditional medicine, exerts an antiproliferative effect on various tumor cell lines by an incompletely understood mechanism. It has been shown that low doses of curcumin downregulate DNA topoisomerase II alpha (TOP2A) which is upregulated in many malignances. The activity of TOP2A is required for RNA polymerase II transcription on chromatin templates. Recently, it has been reported that CTCF, a multifunctional transcription factor, recruits the largest subunit of RNA polymerase II (LS Pol II) to its target sites genome-wide. This recruitment of LS Pol II is more pronounced in proliferating cells than in fully differentiated cells. As expression of imprinted genes is often altered in tumors, we investigated the potential effect of curcumin treatment on transcription of the imprinted H19 gene, located distally from the CTCF binding site, in human tumor cell lines HCT 116, SW 620, HeLa, Cal 27, Hep-2 and Detroit 562. Transcription of TOP2A and concomitantly H19 was supressed in all tumor cell lines tested. Monoallelic IGF2 expression was maintained in curcumin-treated cancer cells, indicating the involvement of mechanism/s other than disturbance of CTCF insulator function at the IGF2/H19 locus. Curcumin did not alter H19 gene transcription in primary cell cultures derived from normal human tissues. J. Cell. Biochem. 104: 1781–1792, 2008.

Key words: curcumin; H19; DNA topoisomerase II α; tumor cells

Curcumin (diferuloloylmethane), a polyphenol derived from the plant *Curcuma longa*, has long been used as an anti-inflammatory and anti-proliferative agent in traditional medicine. Despite the common belief that natural compounds, used for centuries to treat a wide variety of pathological conditions, are safe and efficacious, the exact definition of their mode of action is often lacking. Curcumin suppresses tumor initiation, progression and metastasis [Aggarwal et al., 2003]. The in vivo and in vitro antitumor properties of curcumin stem from its ability to suppress proliferation of a number of cancer cells by downregulating transcription factors, expression of cyclooxygenase-2 (COX-2), inducible nitric-oxide synthase, matrix matalloproteinase-9, tumor necrosis factor, chemokines, cell surface adhesion molecules, cyclin D1, growth factor receptors, as well as inhibiting the activity of c-Jun NH2 -terminal kinase, protein tyrosine kinases, protein serine/threonine kinases, and DNA topoisomerase II α (TOP2A) [Roth et al., 1998; Bhaumik et al., 1999; Hadi et al., 2000; Kim et al. 2001; Roy et al., 2002; Aggarwal et al., 2003; Martin-Cordero et al., 2003; Aggarwal and Shishodia, 2004].

Although the importance of changes in expression of a number of imprinted genes for carcinogenesis has been well documented [Rainier et al., 1993; Kondo et al., 1995], there are no reports on a possible effect of curcumin on their expression. Imprinted genes are a subset of autosomal genes in diploid organisms that are expressed exclusively from one of the parental alleles [Bartolomei and Tilghman, 1997]. Irregular expression of imprinted genes often occurs

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*Correspondence to: Renata Novak Kujundžić, Laboratory of Molecular Pathology, Division of Molecular Medicine, Ruđer Bošković Institute, Bijenička 54, 10000 Zagreb, Croatia. E-mail: rnovak@irb.hr

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in a variety of tumors [Rainier et al., 1995; Kim et al., 1998; Randhawa et al., 1998]. The most studied cluster of imprinted genes, which includes H19 and Insulin-like growth factor-2 (IGF2), is located on human chromosome 11p15.5 [Paulson et al., 2000]. Increasing evidence has accumulated regarding the essential role of H19, non-coding RNA that behaves as an oncogene, in tumor growth [Matouk et al., 2007]. The expression of H19 is low in most tissues postnatally and is reactivated during adult tissue regeneration and tumorigenesis. The reciprocal imprinting of the maternally expressed H19 and paternally expressed IGF2 genes depends on a differentially methylated, imprinting control region (ICR), situated upstream of the H19 gene [Leighton et al., 1995; Thorvaldsen et al., 1998]. The ICR is, in most human tissues, methylated on the paternal allele and unmethylated on the maternal allele [Zhang et al., 1993; Jinno et al., 1996; Vu et al., 2000]. In humans, this region contains seven CCCTC binding factor (CTCF)-binding sites [Hark et al., 2000] and, when they are unmethylated, CTCF binds and insulates enhancers downstream of H19 from the IGF2 promoter without hindering expression of H19 [Hark et al., 2000; Bell and Felsenfeld, 2000; Kaffer et al., 2000; Kanduri et al., 2000]. The unmethylated state of ICR is necessary for CTCF binding, but it is not sufficient for either suppression of IGF2 transcription [Ulaner et al., 2003] or promotion of H19 expression [Manoharan et al., 2004]. Other factors, besides the methylation of ICR, also play important roles in regulation of IGF2 and H19 gene expression. The activity of poly (ADP-ribose) polymerase 1 (PARP1) has been shown to be indispensable for establishment of the ICR-CTCF insulator complex [Klenova and Ohlsson, 2005]. Other partner molecules that interact and cooperate with CTCF in regulation of transcription are being characterized. The largest subunit of RNA polymerase II (LS Pol II) has been recently reported to co-localize with a subpopulation of CTCF genome-wide [Chernukin et al., 2007]. In light of a report that the activity of TOP2A, known to be downregulated by curcumin [Martin-Cordero et al., 2003], is necessary for RNA polymerase II transcription on chromatin templates [Mondal and Parvin, 2001], we hypothesized that curcumin treatment might also attenuate H19 gene transcription.

Topoisomerase II α is an ATP-dependent nuclear enzyme that changes the topological structure of DNA through formation of a complex with DNA, subsequently creating a transient double strand break allowing for DNA replication, repair and transcription [Wang, 1981; Ross, 1985]. The expression of TOP2A is dependent on the state of cellular proliferation, being high in highly proliferating cells and low in quiescent ones. Accordingly, the level of this enzyme varies during the cell cycle, with the highest level at the G_2/M phase and the lowest after completion of the M phase [Kroll and Rowe, 1991]. High levels of TOP2A have been observed in a wide variety of tumors [McLeod et al., 1994; Holden et al., 1995; Dingemans et al., 1998]. The expression of TOP2A is influenced by the rate of protein synthesis, initial posttranslational modification, proper folding and maturation of newly synthesized transmembrane and secretory proteins in endoplasmic reticulum (ER) through expression of the 78 kDa glucose-regulated protein 78 (GRP78) [Gosky and Chatterjee, 2003].

Stress conditions, including nutrient deprivation and hypoxia that are often observed in tumors, lead to protein misfolding with consequent accumulation and aggregation of unfolded proteins in the ER. Cells respond to stress by a number of processes collectively termed the unfolded protein response (UPR) [Zhang and Kaufman, 2006]. Three ER-localized transmembrane proteins Ire1, PERK, and ATF6 are sensors of changes in the ER environment. In the absence of stress, the ER chaperone GRP78 binds to the luminal portions of these proteins and maintains them in their inactive form [Bertolotti et al., 2000]. When unfolded proteins start to accumulate, the GRP78 is released from the transducers resulting in dimerization and activation of Ire1 and PERK or transport of ATF6 to the Golgi [Bertolotti et al., 2000; Shen et al., 2002]. Ire1 is an endoribonuclease that, when activated, alters the reading frame of transcription factor XBP-1, involved in transcription of EDEM, which is a component of the ER degradation machinery [Yoshida et al., 2003]. PERK is a member of the eIF-2α kinase family [Shi et al., 1998] that upon phosphorylation prevents the formation of translation initiation complexes, and thus blocks protein synthesis. ATF6, upon its dissociation from ER membrane, translocates to the nucleus where it activates transcription of its

targets, including GRP78 and XBP-1 [Ye et al., 2000; Yoshida et al., 2001]. Thus, the primary purpose of UPR activation during ER stress is protection of cells through: (1) downregulation of protein translation to prevent accumulation of misfolded proteins; (2) transcriptional upregulation of ER chaperons and folding proteins; (3) propagation of misfolded protein degradation. When cells are unable to adapt to persistent stress, the UPR triggers apoptosis [Faitova et al., 2006]. The mechanisms by which tumor cells, which rapidly proliferate in an environment of sub-optimal nutrient and oxygen availability, take advantage of the cytoprotective elements of the UPR and avoid cytotoxic ones have not been elucidated.

Tumors, due rapid cell proliferation, often have insufficient supply of nutrients, including concommitant shortage of NAD precursor niacin/nicotinamide and essential amino acids. Downregulation of NAD level by 6-aminonicotinamide (6AN) has been reported to upregulate GRP78 and downregulate TOP2A [Gosky and Chatterjee, 2003]. Essential amino acid insufficiency, on the other hand, has been reported to cause ADP-ribosylation and consequential inactivation of GRP78 [Leno and Ledford, 1990]. ADP-ribosylation of GRP78 is accompanied with downregulation of eIF-2 phosphorylation and GRP78 transcription [Laitusis et al., 1999]. This may be of relevance to sustained proliferation of tumor cells under stress conditions since many tumor cells and tumor infiltrating dendritic cells express indoleamine 2,3-dioxygenase (IDO), the first enzyme in the kynurenine pathway of tryptophan degradation which leads to local depletion of this essential amino acid. The immunosuppressive effect of IDO-mediated, local tryptophan depletion has been shown within tumors and tumor-draining lymph nodes, but the consequences of IDO expression on tumor growth are poorly understood [Munn, 2006]. It has recently been reported that COX-2, often highly expressed in tumors, induces expression of IDO [Basu et al., 2006]. Although there are no reports on negative effect of curcumin on IDO, the downregulation of COX-2 by curcumin has been well documented [Rao, 2007].

The aim of this study was to determine the effect of curcumin, a known negative regulator of TOP2A, on H19 transcription. Since H19 transcription is mediated by LS Pol II, we postulated that downregulation of TOP2A, which is indispensable for RNA polymerase II transcriptional activity on chromatin templates, would result in downregulation of H19 transcription. First, we set out to investigate a mechanism responsible for higher expression of TOP2A and H19 in tumors compared to normal tissues. Cognizant of the inverse relationship between expression of GRP78 and TOP2A, we questioned why GRP78 would be less active in tumors. Among stress factors known to influence GRP78 expression is deficiency of essential amino acids, which causes its ADP-ribosylation and consequent inactivation. Since many tumors and tumor-infiltrating immune cells express IDO which mediates tryptophan degradation and leads to its local depletion, this seemed to be a logical mechanism responsible for downregulation of the active pool of GRP78, elevation of TOP2A expression and subsequently LS Pol II-mediated H19 transcription. We show here that all tested tumor cell lines constitutively express IDO, as well as high level of TOP2A and H19 and that their expression at transcriptional level is downregulated by curcumin treatment.

MATERIALS AND METHODS

Cell Culture and Treatment

Human colon carcinoma cell line HCT 116, heterozygous for IGF2, was grown in RPMI-1640 medium with 10% FBS, antibiotics and 5% CO_2 at 37°C. Cells were grown in 24-well plates (2×10^6 cells/ml) for 24 h, the culture medium was removed and replaced with new medium containing 20 μM curcumin, 0.5 μM 5-azacytidine (5-aza H), 0.1 μM 5-azacytidine (5-aza L) or a combination of 20 μM curcumin and either 5-azaH or 5-azaL. The 5-azacytidine (AzaC) is a DNA demethylating agent that inhibits cell growth and induces apoptosis in certain cancer cells. Cells were cultured 48 h before harvesting. Primary cell cultures were prepared from human thyroid tissue, skin and tonsils, obtained during surgery from patients with conditions unrelated to cancer, using Tissue Cell Dissociation Kit (Sigma). Tissues were obtained with previously written informed consent. All primary cell cultures were tested for IGF2 informativity. The primary thyroid cell culture heterozygous for IGF2 was used as control. Cells were grown and treated under the same conditions as HCT116 cells.

Several other human tumor cell lines of different origin: SW 620 (lymph node metastasis, colon adenocarcinoma), HeLa (cervical epitheloid carcinoma,), Cal 27 (tongue, carcinoma), Hep-2 (laryngeal epidermoid carcinoma, HeLa markers) and Detroit 562 (pharyngeal carcinoma) were grown in DMEM medium with 10% FBS, antibiotics and 5% CO_2 at 37°C. Cells were grown in 24-well plates (2×10^6 cells/ml) for 24 h, the culture medium was removed and replaced with new medium containing 30 μM curcumin (SW 620), 40 μM curcumin (Cal 27), 50 μM curcumin (HeLa, Detroit 562, Hep-2) or a combination of respective concentrations of curcumin and either 0.5 μM 5-azacytidine (5-azaH) or 0.1 μM 5-azacytidine (5-azaL). Cells were cultured 48 h before harvesting.

Genomic DNA and Total RNA Extraction

Genomic DNA was extracted from 2×10^6 cells by phenol–chloroform method [Sambrook et al., 1989]. Total RNA was extracted using RNA-Bee reagent (Biogenesis Ltd., Poole, England) according to manufacturer's instructions. It was further purified using RNeasy® Mini kit (Qiagen, Hilden, Germany). To remove traces of contaminating DNA, the RNA was treated with DNase using RNase-free DNase set (Qiagen). The integrity of RNA was determined by electrophoresis on a 1% agarose gel.

Determination of IGF2 Zygosity

Determination of IGF2 zygosity in genomic DNA was based on the *ApaI* polymorphism. Briefly, primers ApaIF: 5′-CTTGGACTTTGAAGTCAAATTGG-3′ and ApaIR: 5′-ATCGTTGTTGGGTCTGACCGAGGAG-3′ were used to amplify a 173 bp long segment of exon 9 from genomic DNA. The reaction was performed in a 25 μl reaction mixture containing dNTPs (50 μM each), 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 8 pmol of each primer and 0.25 U of rTaq polymerase (TaKaRa). The reaction proceeded in 38 cycles composed of denaturation at 95°C for 30 s, annealing at 57°C for 30 s and primer extension at 72°C for 30 s (+1 s in every subsequent cycle). The PCR product was digested overnight with 10 U *ApaI* endonuclease (Roche) at 37°C, yielding an intact 173 bp fragment (homozygote—uncut) or 173, 108 and 65 bp fragments (heterozygote—one allele cut, another uncut) or 108 and 65 bp fragments (homozygote—cut). The digested PCR products were electrophoresed through 8% polyacrylamide gel and silver stained [Mitchell et al., 1994].

RT-PCR

One microgram of total RNA was reverse transcribed using 0.5 μg/μl oligo $(dT)_{18}$ (New England BioLabs), 0.5 mM dNTPs, 20 U of RNase inhibitor (Roche), 1× incubation buffer (supplied with M-MuLV enzyme: 75 mM KCl, 50 mM Tris–HCl, 3 mM $MgCl_2$, 10 mM dithiotreitol; pH 8.3) and 40 U of M-MuLV reverse transcriptase (New England BioLabs) in total volume of 20 μl. The reaction proceeded for 10 min at 70°C followed by 1 h incubation at 37ºC. The quality of cDNA was evaluated by amplifying part of the housekeeping gene GAPDH. The primer sequences for GAPDH were GAPDH1: 5′-AACGGATTTGGTCGTATTGGGC-3′ and GAPDH2: 5′-AGGGATGATGTTCTGGAGAG CC-3′. To exclude the presence of DNA contamination part of GAPDH was amplified using primers GAPDH2 and GAPDH3: 5′-AAGCTGACTCAGCCCGCAAAGG-3′, complementary to intron 5. The 25 μl reaction mixture was composed of dNTPs (50 μM each), 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 6 pmol of each primer and 0.25 U of rTaq polymerase (TaKaRa). The reaction proceeded for 42 cycles with annealing temperature of 56°C. The PCR products were electrophoresed on 1% agarose gel containing SyberSafe, visualized under UV light and photographed.

Successfully transcribed cDNA was used as template in RT-PCR reactions using primers for H19 (forward 5′-CGGACACAAAACCCTCTAG-CTTGGAAA-3′ and reverse 5′-GCGTAATGGAATGCTTG AAGGCTGCTC-3′); IGF2 (forward 5′-CTTGGACTTTGAGTCAATTGG-3′ and reverse 5′-ATCGTTGTTGGGTCT GACCGAGGAG-3′); TOP2A (forward 5′-CACAACTGGCCCTCTCTTCTGCGAC -3′ and reverse 5′-GGGCAACCTTTA CTTCT CGCTT -3′); CTCF (forward 5′-AGGGCATTCAGAACAGTCAC-3′ and reverse 5′-CACTTTGGGTAAACCGAGCATGAC-3′); COX-2 (forward 5′-ATTCTTTGCCCAGCACT TCACG-3′ and reverse 5′-ATC TCTGCCTGAGTATCTTTGACTGTGG-3′); and IDO (forward 5′-AAGGTCATGGAGATGTCC GTAAGG-3′ and reverse 5′-TCCACCAATAGA GAGACCAGGAAG-3′). The PCR products were electrophoresed on 2.5% agarose gel containing SyberSafe, visualized under UV light and photographed.

Measurement of NAD

Pyridine nucleotide content (NAD and NADH) was estimated by the thiazolyl blue micro cycling assay [Bernofsky and Swan, 1973] modified to a microtiter plate format. Briefly, 10^6 cells were homogenized in 1 ml PBS containing PARP inhibitor by sonication for 30 s on ice at 100 W. A reaction mixture (125 μl), composed of ethanol (120 μmol/ml), alcohol dehydrogenase (1 mg/ml), phenazine methosulfate (1 mg/ml), and MTT (0.2 mg/ml) in bicine-buffered solution (pH 7.8), was added to 20 μl of cell homogenate. Alcohol dehydrogenase converted available NAD+ in the sample to the reduced form (NADH). Formazan dye was formed due to reduction of MTT by NADH. The reaction proceeded in the dark for 10 min at 37°C and was stopped by addition of 125 μl of 12 mM iodoacetic acid. Absorbance was read at 550 nm in a microtiter plate reader.

Cell Viability Assay

Cells were seeded, at 1×10^4 cells/well, in 96-well plates and treated with curcumin (0, 10, 20, 30, 40, 50 μM) for 24, 48 and 72 h. Thereafter, 20 μl of 50 mg/ml MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] were added to each well and the plates were incubated for 3 h at 37°C. Formazan crystals were dissolved by addition of DMSO and the absorbances were read at 550 nm using a plate reader. Results were expressed as percentage loss of cell viability compared with corresponding untreated cells.

RESULTS

Curcumin Decreases Tumor Cell Viability

To determine the effect of curcumin on the HCT 116, SW 620, HeLa, Cal 27, Hep-2 and Detroit 562 cell viability, cells were treated with 0–50 μM curcumin for 24, 48 and 72 h and assayed with MTT. The normal human diploid fibroblasts Wi-38 were used to ascertain that antiproliferative effect of curcumin is tumor-specific. Curcumin inhibited cell viability of all tested tumor cell lines but not the viability of normal human fibroblasts in dose- and time-dependent manner (Fig. 1). The curcumin concentrations that caused approximately 50% inhibition of cell viability after 48 h treatment were used in subsequent experiments.

Curcumin Downregulates H19 and TOP2A Transcription Only in Tumor Cells

To determine whether curcumin, as a TOP2A inhibitor, would influence transcription of the imprinted H19 gene whose transcription is RNA polymerase II-dependent, HCT 116 cells were treated with curcumin, 5-azaH, 5-azaL or combinations of curcumin and 5-aza, as described earlier. The expression of H19, IGF2, CTCF and TOP2A was analyzed by RT-PCR. While 5-aza treatments slightly enhanced H19 expression in HCT116 cells, it was completely

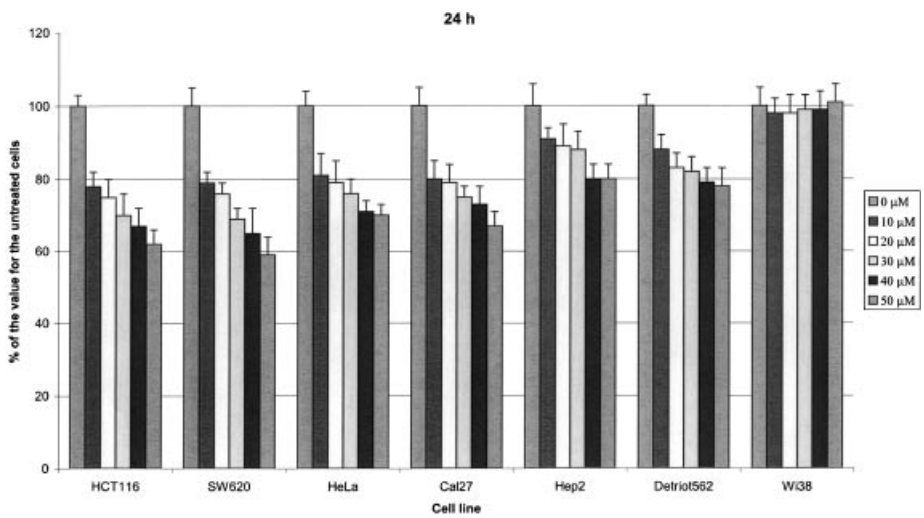

Fig. 1. Curcumin inhibits tumor cells viability. Cells were treated for 24, 48, and 72 h with 0–50 μM curcumin and analyzed by the MTT cell-proliferation assay. The mean ± standard error values of six samples are shown. Optical density values were adjusted to % control values (with no curcumin = 100%). This figure shows that curcumin inhibits tumor cells viability in a concentration- and time dependent-manner.

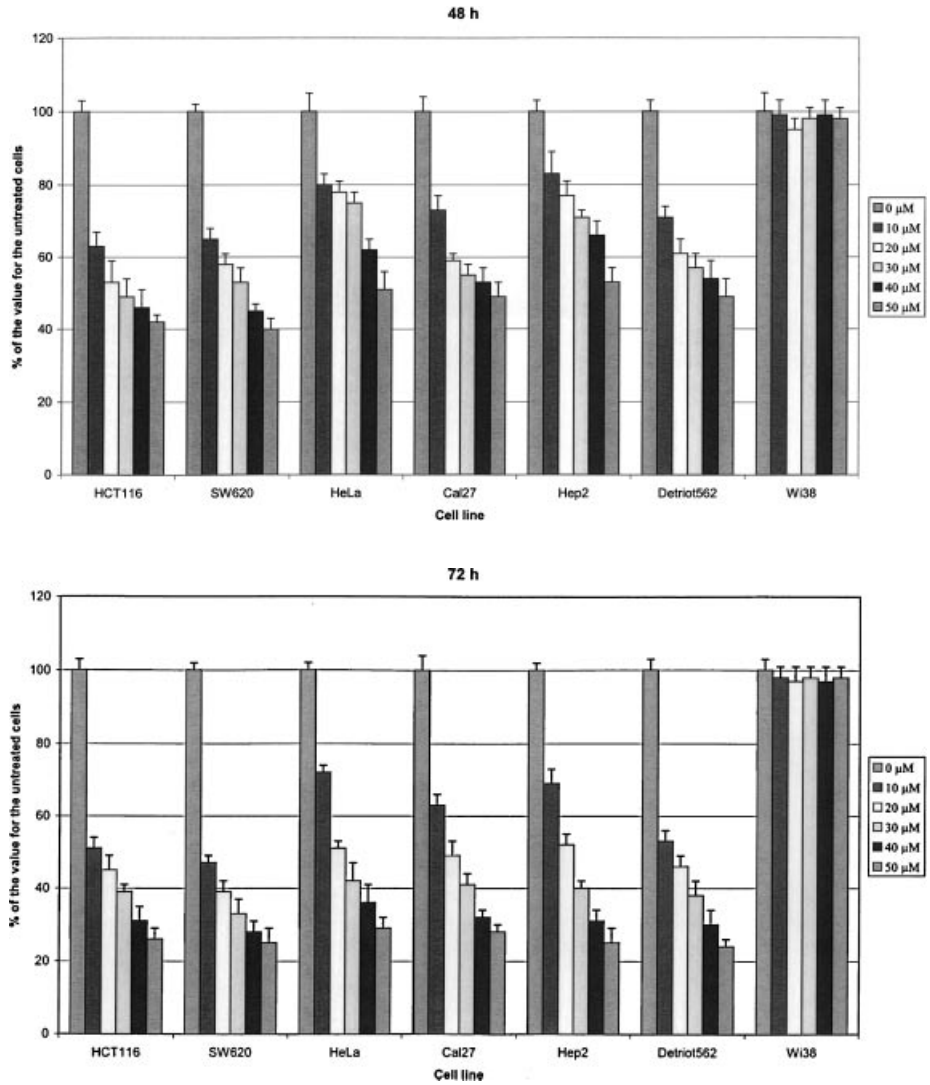

Fig. 1. (*Continued*)

silenced upon curcumin treatment, but only in the tumor cells (Fig. 2A). The expression of H19 was maintained at the comparable low level in untreated and curcumin-treated-non-cancerous thyroid cells (Fig. 2B).

Both cell types were selected for this study based on their monoallelic expression of IGF2, which implies that the insulator property of ICR-CTCF complex was maintained. Indeed, curcumin treatment did not cause any elevation in IGF2 that would accompany H19 silencing due to ICR-CTCF insulator loss of function. Even more, none of these treatments affected the CTCF transcription, indicating that some other mechanisms may be responsible for curcumin induced H19 silencing in tumor cells.

In order to execute its transcriptional activity, CTCF must associate with LS Pol II. This process is dependent on TOP2A, which was significantly downregulated in the cancer cell line upon curcumin treatment.

It is well known that 5-aza demethylates the ICR, which leads to CTCF binding and H19 transcription. When combined with curcumin, 5-aza cannot exert this effect (Fig. 2A). We hypothesize that, in this particular combination, 5-aza still demethylates the ICR, but H19 transcription does not proceed due to decreased activity of LS Pol II, which is TOP2A dependent.

To ascertain that curcumin-induced downregulation of TOP2A and consequently H19 transcription is characteristic for tumor cells, regardless of origin and growth characteristics, we analyzed the expression of those genes in SW 620, HeLa, Cal 27, Hep-2 and Detroit 562 tumor cells treated with curcumin alone or in

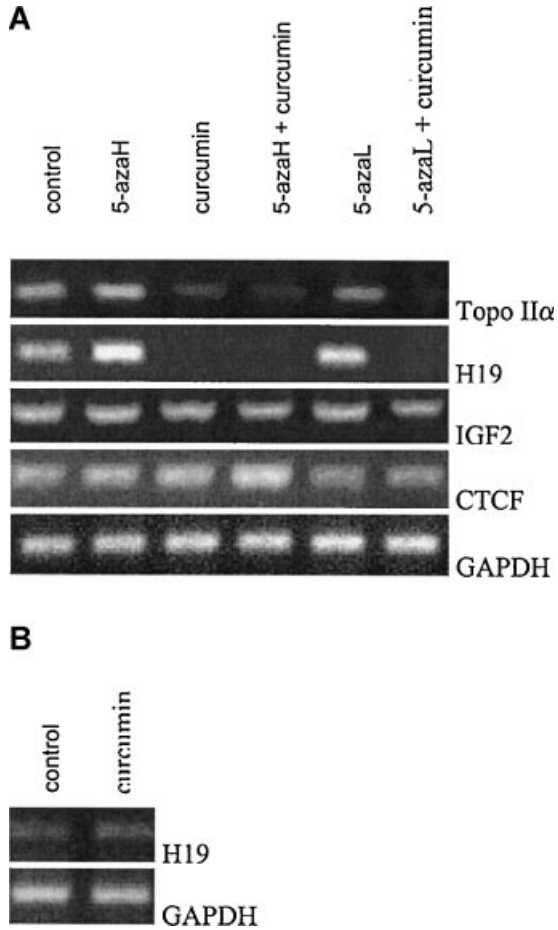

Fig. 2. Curcumin abolishes H19 transcription and downregulates TOP2A in HCT116 cells. **A**: Expression of H19, IGF2, TOP2A and CTCF in HCT116 cells (2×10^6 cells/ml), treated with 20 μM curcumin, 0.5 μM 5-azacytidine (5-aza H), 0.1 μM 5-azacytidine (5-aza L) or their combination and cultured for 48 h. **B**: Expression of H19 and TOP2A in primary normal thyroid cell culture treated with 20 μM curcumin for 48 h.

combination with 5-azaL or 5-azaH. Upon above listed treatments, TOP2A transcription was downregulated and H19 was completely silenced in SW620 cells, similar to HCT116 cells. Transcription of TOP2A was completely silenced in HeLa cells by curcumin (50 μM) or combined treatment with 5-aza, accompanied with downregulation of H19 transcription. The carcinoma of the tongue cell line Cal 27 was less sensitive to curcumin treatment in regard to TOP2A expression. Those cells had very high basal expression of TOP2A, which was downregulated after treatment with 40 μM curcumin and combination of 40 μM curcumin and 5-azaL or 5-azaH. The transcription of H19 was completely silenced in Cal 27 cells upon treatment with curcumin alone and downregulated by combined treatment with curcumin and 5-aza. The laryngeal carcinoma cell line, Hep-2, responded to curcumin treatment (50 μM) by complete silencing of TOP2A and its downregulation upon combined treatment with curcumin and 5-aza. The transcription of H19 was only slightly downregulated in this cell line by all listed treatments. The expression of TOP2A gene in pharyngeal carcinoma cell line Detroit

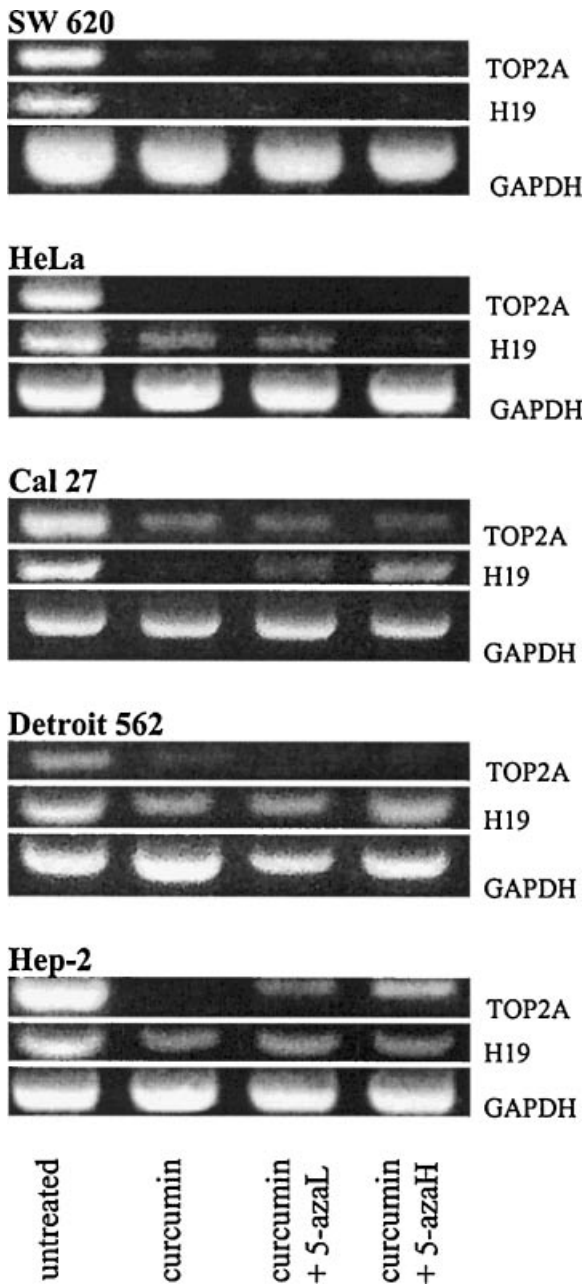

Fig. 3. Curcumin downregulates TOP2A and H19 transcription in different tumor cell lines. Expression of TOP2A and H19 in SW620, HeLa, Cal27, Hep-2 and Detroit562 cells upon treatment with curcumin alone or in combination with 0.5 μM 5-azacytidine (5-aza H) or 0.1 μM 5-azacytidine (5-aza L) for 48 h.

562 was downregulated by treatment with 50 μM curcumin and completely silenced by combined treatment with curcumin and 5-azaL or 5-azaH, but it was accompanied with only slight downregulation of H19 transcription after 48 h (Fig. 3).

Curcumin Downregulates Transcription of COX-2 and IDO in Tumor Cells

To determine the possible relationship between TOP2A and IDO expression, both IDO and COX-2, which Basu et al. [2006] have reported to potentiate IDO expression, were analyzed by RT-PCR. HCT 116 cells constitutively express low levels of IDO and COX-2 (Fig. 4A). Treatment with 20 μM curcumin caused complete silencing of both COX-2 and IDO expression. All other tested tumor cells constitutively express IDO. Their response to curcumin treatment was either downregulation of IDO transcription (Cal 27, Detroit 562, Hep-2) or its complete silencing (SW 620, HeLa) (Fig. 4B). Neither COX-2 nor IDO transcripts were detected in cells derived from non-malignant thyroid tissue.

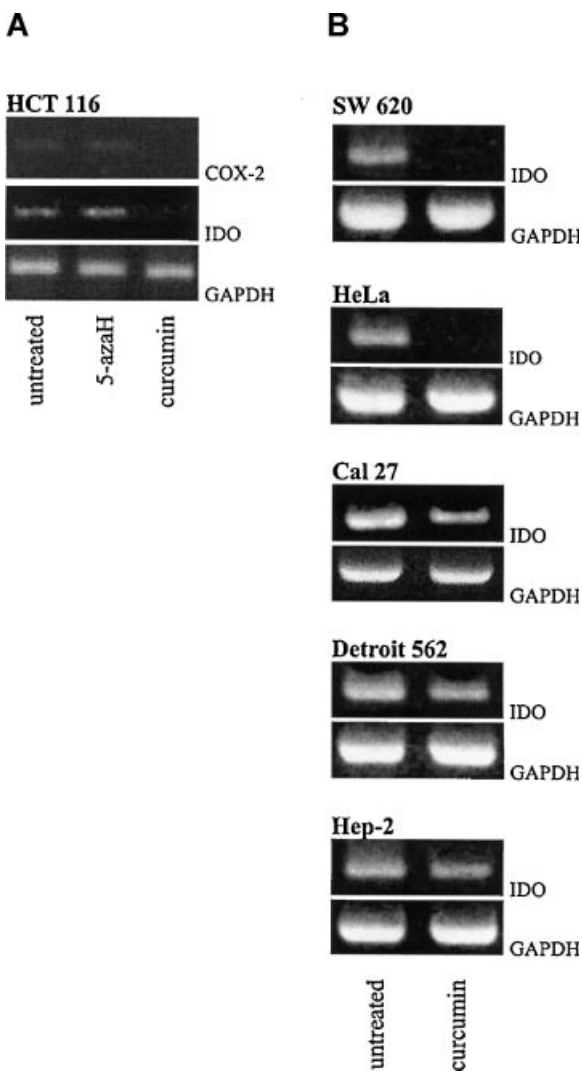

Fig. 4. Curcumin downregulates transcription of COX-2 and IDO. **A**: Expression of IDO and COX-2 in HCT116 cells (2×10^6 cells/ml), treated with 0.5 μM 5-azacytidine (5-aza H) or 20 μM curcumin for 48 h. **B**: Expression of IDO in untreated and curcumin-treated SW 620, HeLa, Cal 27, Detroit 562 and Hep-2 cells.

IGF2 Monoallelic Expression Is Maintained Upon Treatment With Curcumin

To ascertain that curcumin treatment affected only H19 transcription without disturbing insulator function of the ICR-CTCF complex, we analyzed allelic expression of IGF2 in HCT116 and in non-malignant cells that were treated as described previously. Both cell types maintained monoallelic expression of IGF2 regardless of type of treatment (Fig. 5) and status of H19 expression.

Curcumin Treatment Increases NAD Level in Tumor Cells

The level of NAD+ was measured to determine weather it reflects the difference in response of tumor and non-malignant cells to curcumin treatment. The concentration of NAD+ in analyzed tumor cell lines SW 620, HCT 116, HeLa, Cal 27, Hep-2 and Detroit 562 varied and was 9%, 25%, 34%, 46%, 47%, and 57% of the concentration in normal human dioploid fibroblast cell line Wi-38 respectively. Curcumin has been reported to cause degradation of PARP [Mukherjee Nee Chakraborty et al., 2007]. To determine to which extent curcumin-mediated NAD upregulation could be attributable to inhibition of PARP or other ADP-ribosylating reactions, cells were treated with

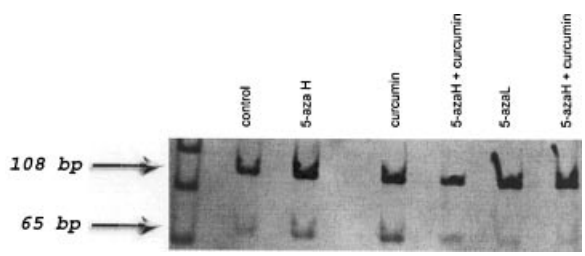

Fig. 5. Monoallelic expression of IGF2 in HCT116 cells (2×10^6 cells/ml), treated with 20 μM curcumin, 0.5 μM 5-azacytidine (5-aza H), 0.1 μM 5-azacytidine (5-aza L) or their combination for 48 h.

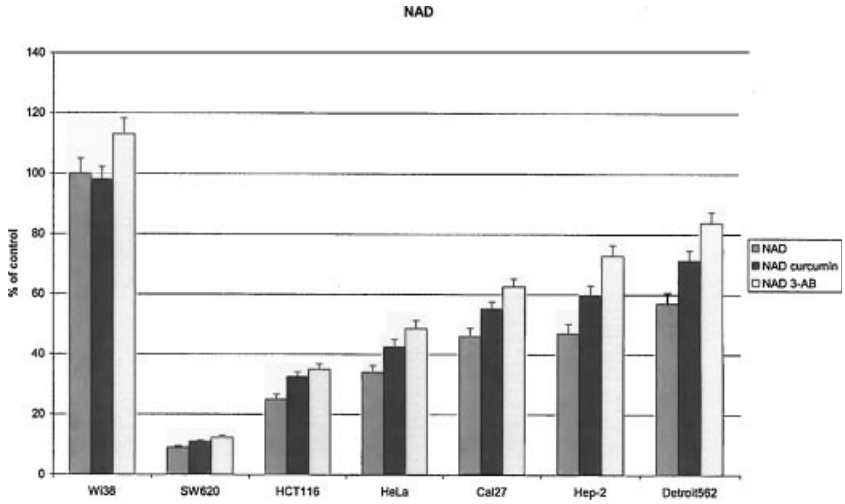

Fig. 6. NAD levels in Wi-38 (normal diploid fibroblasts) and tumor cells SW620, HCT116, HeLa, Cal27, Hep-2 and Detroit562. Cells (2×10^6 cells/ml) were treated for 48 h with either 20 μM curcumin (HCT116), 30 μM curcumin (SW620), 50 μM curcumin (Wi38, HeLa, Cal27, Hep-2 and Detroit562) or 100 μM 3-aminobenzamide. Values are expressed as % of control value measured in untreated cells and are mean ± SE.

20 μM curcumin or with the widely used PARP inhibitor 3-aminobenzamide (3-AB). Treatment of SW 620, HCT 116, HeLa, Cal 27, Hep-2 and Detroit 562 cells with curcumin caused an increase in NAD+ level of 21%, 30%, 25%, 20%, 27%, and 25%, respectively, compared to untreated cells. Chemical inhibition of PARP activity by 100 μM 3-AB elevated NAD+ concentration by 37%, 40%, 43%, 36%, 55%, and 47%,respectively, compared to untreated cells (Fig. 6). The differences in NAD level between control, untreated cells and treated cells, regardless of treatment, were statistically significant according to Student's *t*-test ($P < 0.05$).

DISCUSSION

In this study, we have examined the potential effect of curcumin-induced downregulation of TOP2A on H19 transcription in HCT116, SW 620, HeLa, Cal 27, Hep-2 and Detroit 562 tumor cells. Our results show that TOP2A is constitutively expressed in all studied tumor cells at higher level than in cells derived from non-malignant tissue. Curcumin treatment downregulated the constitutively, highly expressed TOP2A in tumor cells but had no effect on its expression in non-malignant cells. Greater abundance of TOP2A mRNA in tumor cells than in non-malignant cells has been described [McLeod et al., 1994; Holden et al., 1995], but the reason for a difference in the expression level of TOP2A between tumor and non-tumor cells has not been explained. The report of an inverse relationship between expression of TOP2A and GRP78 has shed some light into regulatory mechanisms of TOP2A expression [Gosky and Chatterjee, 2003]. The GRP78 is constitutively expressed in all cells, but can be elevated by various ER stress-inducing factors. It has been reported that HCT116 cells have a low basal level of GRP78 that can be upregulated by 6-aminonicotinamide, an NAD antagonist and weak inhibitor of PARP [Belfi et al., 1999]. The expression of IDO, the rate limiting enzyme of tryptophan degradation in the kynurenine pathway, in tumor cells is yet another possible regulatory mechanism of GRP78 expression and activity, with consequent influence on TOP2A expression in those cells. Under conditions in which cells are deprived of essential amino acids, like tryptophan, GRP78 is mono-ADP-ribosylated, rendering it functionally inactive [Leno and Ledford, 1990]. This elevated proportion of the non-functional form of GRP78 in IDO-expressing tumor cells may explain the higher expression of TOP2A, compared to non-cancerous cells. The expression of IDO is under the influence of COX-2 [Basu et al., 2006], which has been reported to be downregulated by curcumin treatment [Rao, 2007]. In our experimental system, treatment with curcumin caused downregulation of both COX-2 and IDO in HCT 116 cells. This prevents

deprivation of tryptophan, subsequent mono-ADP-ribosylation of GRP78, and thus causes elevation in functional GRP78 in the cell, resulting in downregulation of TOP2A. In contrast to all analyzed tumor cells, we have not detected IDO transcripts in cells derived from non-malignant human thyroid tissue. This is in accord with their unresponsiveness to curcumin treatment, regarding expression of TOP2A.

Concomitant with downregulation of TOP2A, we have observed silencing of H19 in HCT 116, SW 620 and Cal 27 cells, and downregulation of its transcription in HeLa, Hep-2 and Detroit 562 cells. The monoallelic expression of IGF2 was maintained in curcumin-treated HCT 116 cells, suggesting that, despite H19 silencing, CTCF/ICR insulator function was not disturbed. A complete lack of H19 transcription is characteristic for adult liver where it has been shown by ChIP analysis that CTCF associates with two CTCF binding sites in the upstream region of the H19 gene that are homologous in rat, human and mouse [Manoharan et al., 2004]. In the same study, the insufficiency of CTCF association with this region for H19 transcription was further documented by demonstration that in adult liver, in contrast to fetal and neoplastic liver where H19 is expressed, RNA polymerase II is not associated with the first exon region of H19 gene. Liver is the main site of de novo synthesis of NAD from tryptophan [Schutz and Feigelson, 1972] and thus specific in regard to GRP78 expression. Reported high constitutive GRP78 expression in liver [Tillman et al., 1996], involved in downregulation of TOP2A, is in agreement with reported lack of expression of TOP2A in adult murine liver [Capranico et al., 1992].

We further measured the concentration of NAD in tumor and non-malignant cells, untreated and treated with curcumin, to determine if NAD level could be related to changes in TOP2A expression by its influence on GRP78. Non-malignant cells had approximately 4, 11, 3, 2, 2, 2 times higher concentration of NAD than HCT 116, SW 620, HeLa, Cal 27, Hep-2 and Detroit 562 cells, respectively. Treatment of cells with curcumin increased NAD concentration in SW 620, HCT 116, HeLa, Cal 27, Hep-2 and Detroit 562 cells by 21%, 30%, 25%, 20%, 27%, and 25%, respectively, probably as a consequence of downregulation of PARP1 by curcumin [Mukherjee Nee Chakraborty et al., 2007] and possibly other ADP-ribosyltransferases, including those involved in GRP78 ADP-ribosylation. To determine the extent of NAD upregulation that could be attributed to curcumin-induced downregulation of PARP, we treated cells with 3-AB (100 μM), a widely used inhibitor of this enzyme. The level of NAD in 3-AB-treated SW620, HCT116, HeLa, Cal27, Hep-2 and Detroit562 cells increased by 37%, 40%, 43%, 36%, 55%, and 47%, respectively. The observed more pronounced upregulation of NAD level by 3-AB compared to curcumin treatment indicates that curcumin treatment did not cause complete inhibition of PARP activity and possibly other ADP-ribosylating reactions sensitive to 3-AB. Our results suggest that both NAD level of untreated cells and the residual ADP-ribosylating capacity after treatment with curcumin, as estimated from the difference in NAD elevation upon treatments with 3-AB and curcumin, may be a reflection of cellular sensitivity to curcumin-induced downregulation of TOP2A and consequently H19. The observed negative effect of curcumin on proliferation of different tumor cell lines was in accordance with the extent to which it induced downregulation of H19 transcription.

In summary, our results (1) show that curcumin induces downregulation of H19 transcription or even complete silencing of this gene in tested tumor cell lines, (2) indicate that negative effect on H19 transcription is at least partly mediated by downregulation of TOP2A. Our findings also suggest the involvement of NAD metabolism, through ADP-ribosylating reactions, in different sensitivity of non-malignant and malignant cells to curcumin-mediated TOP2A downregulation and consequent inhibition of RNA polymerase II-dependent transcription.

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