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ARTICLES

Alterations in CDK1 Expression and Nuclear/Nucleolar Localization Following Induction in a Spontaneous Canine Mammary Cancer Model

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Abstract Transcription of CDK1 is induced as cells re-enter the cell cycle from quiescence and these early cell cycle re-entry events have been modeled by okadaic acid treatment due to its activity on specific enhancer sequences in the human CDK1 promoter. To investigate heterogeneity of control of this mechanism in the context of neoplastic transformation, a cellular model derived from spontaneous canine mammary cancer (CMT) was developed that includes six cell lines derived from different animals. Notable heterogeneity in response to okadaic acid was observed in expression of CDK1 mRNA and protein. In response to okadaic acid treatment, two CMT cell lines exhibited a CDK1 mRNA induction while one cell line exhibited CDK1 mRNA suppression, and three remained unchanged. Despite this variability, three CMT cell lines arrested in S or G2/M phase and five exhibited marked increases in apoptosis. Moderation of some of these differences were observed at the level of CDK1 protein as three of six CMT cell lines exhibited only moderate enhancement in CDK1 protein levels while three remained essentially unchanged. Some additional differences in distribution of CDK1 protein, favoring enhanced nuclear over cytoplasmic CDK1 localization, were observed in treated cells in the form of concentrated nuclear CDK1 labeled foci. Confocal microscopy revealed the presence of brightly labeled punctate foci containing CDK1 protein within nuclei as well as nucleoli in okadaic acid treated non-mitotic cells suggesting a role for this kinase outside the normal G2/mitotic phase of the cell cycle and suggesting a possible new function within the nucleolus. J. Cell. Biochem. 98: 504–518, 2006.

Key words: cell cycle; CDK1 expression; nuclear/nucleolar location; okadaic acid; canine mammary cancer

The transition through the G2/M phase checkpoint of the cell cycle to mitosis is regulated by the activity of cyclin-dependent kinase 1 (CDK1 or p34/cdc2) protein [Draetta and Beach, 1988; Nurse, 1990; Hunt, 1991; Bird, 1997, 2003; Dahler et al., 1998; Ekholm and Reed, 2000]. CDK1 composes the enzymatic core of the mitotic CDK/cyclin integration complex regulating G2/M transitions [Labbe et al., 1989; Draetta et al., 1989; Gautier et al., 1990; Bird, 1997]. CDK1 is controlled during the continuous cell cycle principally at the level of post-translational modification and through its association with its cofactors cyclin and the p21/Cip1/Waf1 and p27/KIP1 families of CDK inhibitors (CKIs) to form cell cycle phase-specific CDK-based integration complexes [Frey et al., 2001; Bird, 2003]. The function of this enzyme and its cyclin B co-factor is principally to regulate entry into mitosis. However, recent evidence has also suggested that CDK1 may play a role in G1 phase regulation and the apoptotic pathway as well [Itzhaki et al., 1997; Badea et al., 2002; Konishi and Bonni, 2003].

The sub-cellular location of CDK1/cyclin B is critical to proper functioning of this complex [Kong et al., 2000; Takizawa and Morgan, 2000; Ohashi et al., 2001]. During interphase, the CDK1/cyclin B complexes are thought only to reside in the cytoplasm as either a soluble form

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or associated with the microtubule network and the centrosomes [Booher and Beach, 1988; Takizawa and Morgan, 2000]. In late prophase, most of the CDK1/cyclin B complexes are rapidly transported into the nucleus and only a small number of CDK1/cyclin B complexes remain in the cytoplasm associating with duplicated centrosomes as they separate. However, recent experiments have suggested that CDK1/cyclin B complexes shuttle in and out of the nucleus continuously, indicating that the steady-state localization of CDK1/cyclin B complexes may be determined by relative rates of nuclear import and export [Kishimoto and Okumura, 1997].

Cytoplasmic localization of CDK1/cyclin B complexes has been attributed to a 42-residue region in the amino-terminal half of cyclin B called the cytoplasmic retention sequence or CRS [Takizawa and Morgan, 2000]. CRS deletion from cyclin B resulted in localization of cyclin B to the nucleus throughout the cell cycle. Neither CDK1 nor cyclin B contains a basic nuclear localization signal (NLS) or other identifiable import signal. However, situated within the CRS is a leucine-rich nuclear export signal (NES) that has been shown to bind the Crm1 export receptor through phosphorylation of four of five serine residues that are required for appropriate nuclear import/export [Takizawa and Morgan, 2000]. By comparison, CDK2 and CDK4 also do not contain NLS import signals and apparently rely on bound Cip/KIP molecules to provide information necessary for nuclear localization [LaBaer et al., 1997; Weiss, 2003]. There is evidence that CDK1 may require at least p21/Cip-1 for nuclear accumulation and subsequent dissociation of p21/Cip-1 may be required for activation [Charrier-Savournin et al., 2004].

In contrast to post-translational regulation exerted during the continuous cell cycle, regulatory control during the G0/G1 phase transition, as cells re-enter the cell cycle, appears to be exerted at a completely different level. As cells make the transition from G0 to G1 phase, CDK1 transcription is induced from very low to high levels and only later resolves to the constitutive levels of expression observed during sequential phases of the continuous cell cycle [You and Bird, 1995; Onishi et al., 1997]. Thus, as cells re-enter the cell cycle from G0/quiescence, CDK1 levels are regulated primarily by transcription, which is very different from the controls exerted once cells are proliferating and approaching the mitotic checkpoint. We have characterized the G0/G1 phase transcription activation of CDK1 expression and modeled the earliest events with the phosphatase 1/2A inhibitor okadaic acid [You and Bird, 1995; Liu and Bird, 1998; Bird and DeInnocentes, 2004]. Okadaic acid selectively suppresses phosphatase 1/2A activity resulting in hyper-phosphorylation of cellular proteins and possibly enabling proliferation signals through activation of signal transduction [Yamashita et al., 1990; Schonthal, 1992]. This likely perturbs/stimulates signal transduction systems, perhaps mimicking the response of selective signaling components in the normal response to growth factor stimulation. The model is typified by rapid induction of CDK1 expression and other gene products associated with initiation of the cell cycle and represents a less complex reaction than traditional starvation/refeeding.

Canine mammary tumor (CMT)-derived cell lines make an excellent model system in which to study CDK1 expression as there are many similarities between humans and dogs with respect to the development of mammary cancer. Mammary cancers in both species develop spontaneously and are due to somatic mutations [MacEwen et al., 1982; Wolfe et al., 1986; MacEwen, 1990]. Mammary carcinomas are the most common malignancy of female dogs although the incidence of mammary tumors is three times higher than in women [Fidler and Brody, 1967; MacEwen et al., 1982; Wolfe et al., 1986; MacEwen, 1990]. Other similarities between mammary cancers of women and dogs include adenocarcinoma as the malignant cell type, metastasis and rapid evolution, hormonal dependency and presence of hormonal receptors, and an age-dependent occurrence [Weije et al., 1972; MacEwen, 1990; MacEwen and Withrow, 1996, 2001]. The risk for the development of mammary cancer also decreases the earlier a dog is ovariectomized [Weije et al., 1972; Hamilton et al., 1977; MacEwen, 1990]. Both estrogen and progesterone receptors have been identified in 70% of benign CMTs and 50–60% of all malignant mammary tumors [Fidler and Brody, 1967; Weije et al., 1972; Hamilton et al., 1977; MacEwen et al., 1982; MacEwen, 1990; Sartin et al., 1992, 1993; Rutteman and Misdorp, 1993; MacEwen and Withrow, 1996, 2001]. Approximately 40–50% of all mammary tumors that occur in dogs are malignant. CMTs

also exhibit a high frequency of c-*erb*B-2 overexpression and expression of other tumor-associated antigens [Wang et al., 1995; Whitley et al., 1995; Ahern et al., 1996]. In addition, dogs and humans are the only species that share an unusual level of genetic diversity combined with a wide-spread and well-mixed gene pool [Morell, 1997].

To resolve some of the questions involving early G1 phase regulation of CDK1 and to develop the CMT model, CDK1 expression and its regulation were analyzed in six independently derived CMT cell lines and okadaic acid was used to model early events in G1 phase. Spatial and temporal expression of CDK1 and its mRNA were investigated as was the phase of cell cycle arrest in each cell line.

MATERIALS AND METHODS

Cell Culture and Okadaic Acid Treatment

HeLa S3 cells were cultured in α-modified Eagle's essential medium (Gibco) with antibiotics (Sigma) and 10% fetal bovine serum (Summit) as previously described [You and Bird, 1995; Liu and Bird, 1998]. CMT cells were grown at 100% humidity and 37°C with 5% CO_2 in Eagle's modification of α-minimal essential medium (α-MEM) with 10% v/v fetal bovine serum (FBS) and antibiotics (Gibco). CMT-derived cell lines (CMT9, CMT12, CMT25, CMT27, CMT28, CMT47) were obtained from Dr. L.G. Wolfe and cultured as described [Wolfe et al., 1986]. Cells were treated with 19 nM okadaic acid (Moana Bioproducts, Hawaii), from a 2.0 μM stock solution, in growth medium for 12–48 h.

Reverse Transcriptase-PCR

Total RNA was extracted from CMT cells cultured in 75 cm^2 flasks using RNA Stat 60 (Tel-Test, Inc.) according to the direction of the manufacturer. The RNA pellet was resuspended in diethylpyrocarbonate-treated water, concentration of RNA determined by absorbance (260) nm and stored at −80°C [Sambrook et al., 1989]. RNA integrity was analyzed (10 μg/lane) on 1.5% agarose gels in 1 × MOPS (4-morpholinepropanensulfonic acid) buffer, pH 7.0 [Sambrook et al., 1989]. The Access RT-PCR kit (Promega) was used according to the manufacturer's instructions. A master mix containing AMV/T*fl* 1 × reverse transcriptase reaction buffer, dNTP mix (0.02 mM of each), 1 mM $MgSO_4$, five units AMV reverse transcriptase, five units T*fl* polymerase, 2% v/v DMSO, 50 pmol of upstream and downstream oligonucleotide primers, and nuclease free water was assembled and added to 1–2 μg RNA template to a total reaction volume of 50 μl. The forward (5′-CCA GAG CGG CCG CAT TCC AAA AGC TCT GGC AAG GCC-3′) and reverse (5′-CCG GAA CGG TCT CGA AAA CCT TAC GCC GGC GAG ACC-3′) primers were designed from the human CDK1 gene sequence (Genbank accession number Y00272) and amplified in a Perkin Elmer 2400 thermocycler. The reaction protocol was: 48°C for 45 min, 2 min at 94°C, and then 30 cycles of 94°C for 30 s, 55°C for 1 min, and 68°C for 2 min. This was followed by 68°C for 7 min and storage at 4°C. Once amplification was complete, a 10 μl aliquot was taken from each tube, 1 μl of ethidium bromide added, and analysis performed on 1% agarose 1 × TBE (Tris-Boric acid-EDTA) gels [Sambrook et al., 1989]. Gels were photographed under illumination with shortwave UV light.

Flow Cytometry

The six CMT cell lines were grown to approximately 60% confluence and growth medium replaced with α-MEM containing 10% FBS and okadaic acid (19 nM/ml). Cells were collected for fixation, in triplicate, for flow cytometry at the times indicated as previously described with modification [Bird, 1998a,b]. Parallel untreated control cultures were also analyzed. Cells were collected by trypsin digestion, washed twice in HBS (HEPES-buffered saline), placed in 15 ml tubes, and centrifuged 4 min at 3,000 rpm. The supernate was discarded and the cells resuspended in 1 ml HBS. Cells were fixed by slow drop-wise addition of 3 ml of 70% ethanol (−20°C) while vortexing followed by incubation on ice for 30 min. Cells were collected by centrifugation at 3,000 rpm for 2 min and resuspended in 300 μl of PBS and stored at 4°C. Finally, 30 min prior to analysis, cells were stained with propidium iodide (PI) by addition of 250 μl of PI staining solution (33 μl of 4.5 mg/ml PI, 400 μl of 20 mg/ml RNase A, and 9.57 ml of DEPC-water) to fixed-cell suspensions and incubated in the dark for 30 min at room temperature followed by analysis on a MoFlo Flow Cytometer (DakoCytomation) and 10,000 cells were counted per sample. Data were analyzed using Summit software (DakoCytomation) to calculate percent apoptotic

(PI-positive sub-G1 phase DNA content) cells and cell cycle phase distribution for each population as previously described [Cheema et al., 2004; Yang et al., 2004].

Immunohistochemistry

CMT and HeLa cell cultures were treated with okadaic acid for 24 h and harvested by trypsin digestion and resuspended in 1 ml HBS. Two hundred fifty micro liters of each cell suspension was mounted on poly-L-lysine coated slides by cytospin (400 rpm, 5 min) and air-dried for 10 min. Cell preparations were fixed in ethanol/acetone (1:1) for 30 s, air-dried for 5 min, and stored desiccated at 4°C. Hydrogen peroxide (3%) was applied for 5 min and slides rinsed with distilled water. Primary antibody (anti-CDK1, Upstate Biotechnology), recognizing the PSTAIR amino acid sequence, was added (1:200 dilution in PBS) and incubated for 1 h at room temperature in a humidified chamber. Slides were rinsed three times with PBS for 3 min. Conjugated secondary antibody (biotinylated rabbit anti-mouse antibody) was then applied to each specimen, and slides were incubated at room temperature for 30 min in a humidified chamber followed by washing five times for 3 min each with PBS. Then, streptavidin conjugated-horseradish peroxidase was applied to cover the specimen, and the slides incubated for 20 min in a humidified chamber at room temperature and washed with PBS five times for 3 min each. To 2 ml of the substrate buffer (acetate buffer/hydrogen peroxide) was added 1 drop of AEC (3% 3-amino-9-ethylcarbazole/ N, N-dimethylformamide) or DAB (3,3′ diaminobenzidine tetrahydrochloride) chromagen, and the chromagen solution was applied to the slides (Dako LSAB). The cells were incubated for 20 min at room temperature (humidified) and washed five times for 3 min each with distilled water. The cells were counterstained in hematoxylin for 3 min and washed with distilled water 10 times. The cells were subsequently mounted with 1 drop glycergel (Sigma), covered with a coverslip, examined under a microscope, and photographed.

Indirect Immunofluorescence and Confocal Microscopy

CMT cells were treated with okadaic acid (19 nM/ml) for 24 h. Media were removed and the cells were trypsinized, diluted 1:4 in HBS, centrifuged at 400 rpm for 3 min, and resuspended in 250 μl of HBS as described above. The cells were mounted on slides by centrifugation in a cytospin centrifuge at 400 rpm for 5 min and air-dried. Cells were fixed in ethanol/acetone (1:1) solution for 30 s, dried for 5 min, and stored desiccated at 4°C. Cells were washed with 10 mM glycine/PBS two times for 5 min each and then washed in 25 mM glycine/PBS for 15 min. All slides were incubated in blocking solution (5 g bovine serum albumin; 250 μl Tween 20; 15 ml bovine calf serum; 14.61 g NaCl in 500 ml PBS) for 30 min at room temperature. Blocking solution was then removed and cells incubated with primary antibody (anti-CDK1 diluted 1:20 in blocking solution) for 1 h humidified at room temperature. Slides were washed in 10 mM glycine/PBS for 5 min five times and secondary antibody (FITC-conjugated anti-mouse IgG diluted 1:20 in blocking solution) was incubated on cells for 1 h humidified in the dark. Cells were then washed in 10 mM glycine/PBS three times for 5 min each, allowed to dry, mounted in DABCO (Sigma Immunochemicals) with a coverslip, and stored at 4°C in the dark until analyzed by fluorescence microscopy. For dual-labeled immunofluorescent staining, CMT cells were indirectly labeled, as described above, except that primary antibodies recognizing CDK1 (anti-CDK1 rabbit polyclonal IgG recognizing the PSTAIR amino acid sequence added at 1:100 in blocking solution, Upstate Biotechnology) were indirectly stained with secondary antibody (green fluorescent Alexa Fluor 488 conjugated goat anti-rabbit IgG, Molecular Probes) and primary antibodies recognizing nucleolin (anti-nucleolin mouse monoclonal IgG added at 1:50 in blocking solution, Upstate Biotechnology) were indirectly stained with secondary antibody (red fluorescent Alexa Fluor 594 conjugated goat anti-mouse IgG, Molecular Probes).

For confocal microscopy, fluorescein-labeled cells were viewed on a Bio-Rad, MRC-1024 laser scanning confocal microscope using a Silicon Graphics Indigo 2×2 workstation running Vital Images Volume Microscopy Workbench software (Voxelview). Sections were recombined to produce 3-D representations (as rendered volumes in perspective) or stereo 3-D images. Cells were scanned with a 2% laser at $100\times$ magnification and focal planes of 2 μm.

In all experiments reported, multiple replicates were included and each experiment was performed at least three times. In experiments

where percentages of cells expressing CDK1 were evaluated, at least 10 fields were counted including 235–269 cells per determination.

RESULTS

Okadaic Acid Alters Expression of CDK1 mRNA in Canine Mammary Cancer Cells

We have previously demonstrated that expression of CDK1 is sensitive to perturbations caused by okadaic acid treatment through transcriptional activation of the CDK1 promoter in HeLa S3 cells at okadaic acid-activated enhancer elements assessed by Northern blots and a variety of promoter function analyses [You and Bird, 1995; Liu and Bird, 1998; Bird and DeInnocentes, 2004]. To determine if this same response could be demonstrated in canine mammary cancer cells, six CMT cell lines were evaluated for CDK1 mRNA expression by RT-PCR following treatment with okadaic acid for 24 h (Fig. 1). RT-PCR analyses of different CMT cell lines could be divided into three distinct expression profile types with respect to CDK1. CMT12, CMT27, and CMT28 cells expressed barely detectable to abundant CDK1 mRNA levels, which essentially did not change following okadaic acid treatment. CDK1 mRNA was below detectable levels in untreated proliferating CMT9 and CMT47 cells but CDK1 mRNA was induced in the presence of okadaic acid although CDK1 became only just detectable in CMT47 cells following treatment. Lastly, CDK1 mRNAs were readily detectable in exponentially growing CMT25 cells but expression was suppressed below detectable levels by okadaic acid treatment.

Okadaic Acid Arrests Canine Mammary Cancer Cells in S and G2/M Phases and Induces Apoptosis

In previous experiments, HeLa cells treated with okadaic acid arrested in a cell cycle-phase specific block at G2/M and S phases [You and Bird, 1995]. To determine if an analogous cell cycle-phase specific block occurred in CMT cell lines, cells treated with 19 nM of okadaic acid were analyzed by flow cytometry for total DNA content. Cell cycle phase distribution and the proportion of sub-2N DNA/apoptotic cells in each population was quantified and compared to untreated cells. Cell cycle phase-specific arrest was observed in S phase in CMT9 and CMT12 cells while only CMT28 cells appeared to arrest in G2/M phase, analogous to HeLa cells, when each was compared to untreated control cultures (Fig. 2). Treatment time to arrest also varied considerably from 12 h in CMT28 and CMT47 cells to 48 h in CMT25 cells (Table I). Similar to data previously reported for HeLa cells, increases in the apoptotic populations (sub-G1 phase with less than 2N DNA content) in okadaic acid treated CMT9, CMT12, CMT25, CMT27, and CMT28 cell lines of between 2.2 and 10.9-fold were observed with

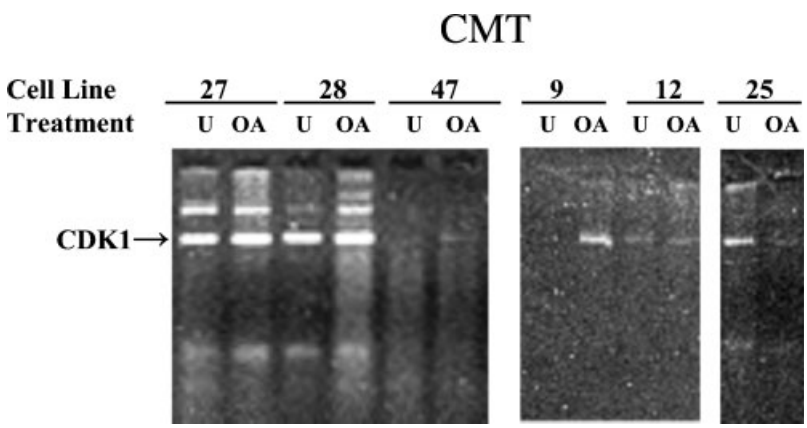


Fig. 1. Expression of CDK1 mRNA in okadaic acid treated CMT cell lines. CMT cells representing six independent CMT cell lines (CMT9, CMT12, CMT25, CMT27, CMT28, and CMT47) were evaluated for CDK1 mRNA expression by RT-PCR assay following treatment with 19 nM okadaic acid (OA), or left untreated (U), for 24 h. Total RNA was isolated and amplified by PCR following reverse transcription. Equal volumes of amplification mix (10 μl/lane) were analyzed by 1% agarose/1 × TBE gel electrophoresis. Arrow indicates the position of the CDK1 amplicon.

TABLE I. Cell Characteristic Changes in Response to Okadaic Acid Treatment

Cell line	Immunohistochemistry	Confocal and high resolution	CDK1	Phase and h to cell cycle
	Immunofluorescence*	Immunofluorescence	mRNA	Arrest/Apoptosis
CMT9	Enhanced cytoplasmic labeling and enhanced nuclear labeling ~20% more cells CDK1 positive	Increased nuclear labeling	Undetectable until induced by okadaic acid	S and apoptotic @ 36 h
CMT12	Little change in labeling levels except enhanced cytoplasmic labeling and enhanced nuclear labeling in ~5% of treated cells		Unchanged by okadaic acid	S and apoptotic @ 36 h
CMT25	Enhanced cytoplasmic labeling with ~40% more cells CDK1 positive		Suppressed by okadaic acid	S and G2/M and apoptotic @ 48 h
CMT27	Enhanced cytoplasmic labeling and enhanced nuclear labeling in ~30% more cells CDK1 positive	Foci localized to nucleoli after okadaic acid (not shown)	Abundant and unchanged by okadaic acid	Apoptotic @ 36 no phase specific arrest
CMT28	Little change in cytoplasmic labeling, nuclear labeling in ~25% of treated cells	Increased nuclear labeling with foci evident foci localized to nucleoli after okadaic acid	Abundant and unchanged by okadaic acid	G2/M and apoptotic @ 12 h
CMT47	Little change in cytoplasmic labeling, nuclear labeling in ~11% of treated cells	Increased nuclear labeling with foci evident	Undetectable until okadaic acid induced trace levels	Arrested @ 12 h no phase specific arrest or enhancement of apoptosis

*Ten fields containing 235–269 cells total were counted for each determination and each experiment was repeated three times.

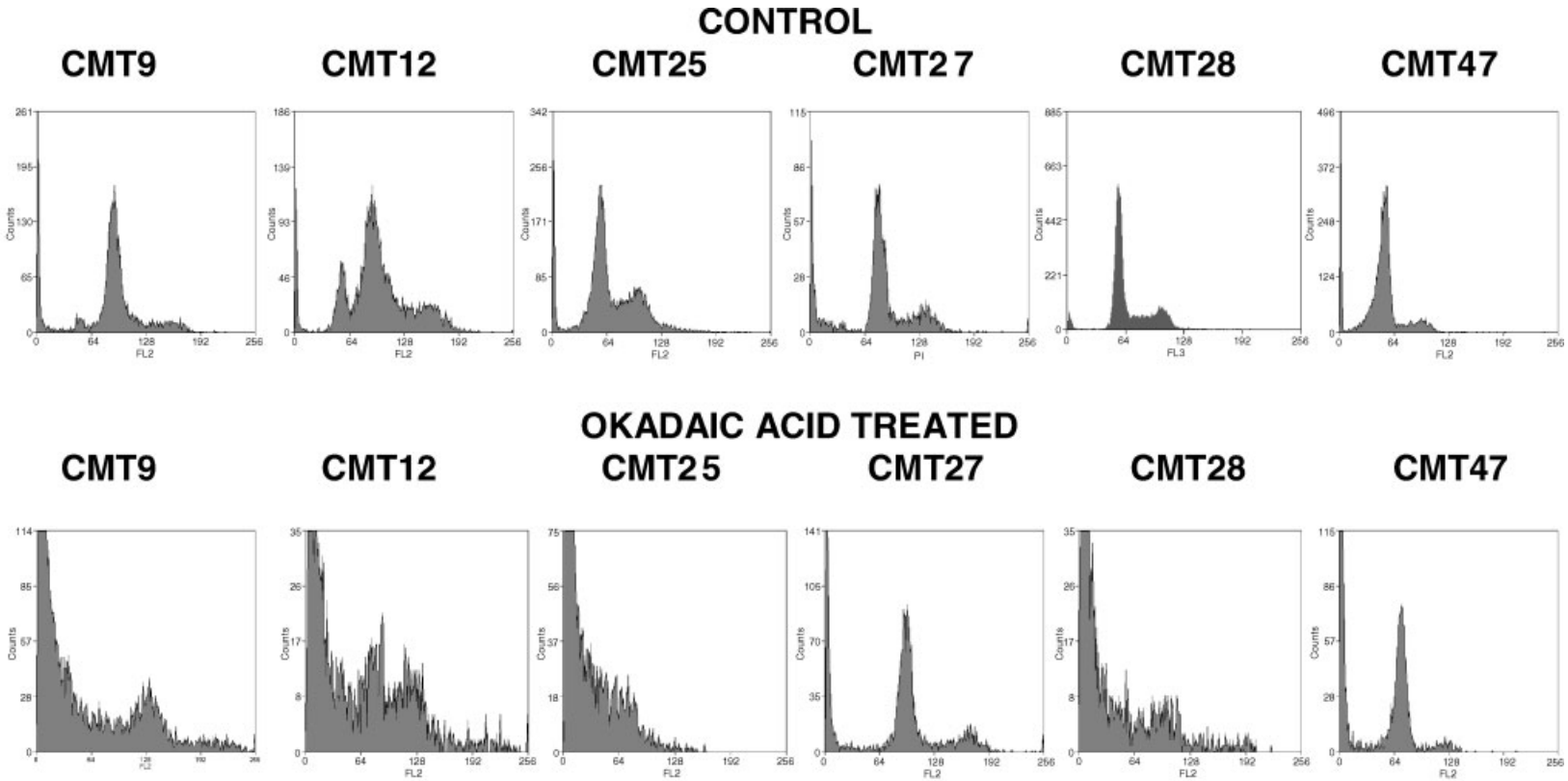
apoptotic cells composing substantial portions of the treated cell populations (41.7–69.3%) in CMT9, CMT12, CMT25, and CMT28 cell lines by 48 h (Fig. 2).

Okadaic Acid Induces Expression of CDK1 Protein in the Cytoplasm and Nucleus

HeLa cells treated with okadaic acid for 24 h expressed a many-fold enhancement of CDK1 protein levels throughout the cytoplasm with uniform and intense staining when compared to control untreated or isotype control labeled cells (Fig. 3). Expression level was estimated as a percentage of levels observed in control untreated cells and, if greatly enhanced above control levels, number of fold above. Enhancement of CDK1 protein levels in HeLa cells was many fold above controls comparable to the greater than 10-fold induction in CDK1 mRNA previously reported [You and Bird, 1995]. CDK1 localization in the nucleus varied from very little to dense accumulations comparable to levels found in the cytoplasm (compare Fig. 3A and 3B). Not all cells expressed large amounts of nuclear CDK1 but the majority of those that did were interphase (non-mitotic) cells. Only low levels of nuclear and cytoplasmic labeling were noted in untreated HeLa cells and no background labeling was evident when an irrelevant isotype control primary antibody was substituted.

The effects of okadaic acid on CDK1 protein levels and localization in CMT cells was not consistent with changes in mRNA levels as assessed by immunohistochemistry (Fig. 4, Table I). When comparing untreated CMT9 cells with okadaic acid-treated CMT9 cells, an increase of approximately 20% was observed in the number of okadaic acid treated cells detected expressing CDK1 (10 fields containing 235–269 cells were counted for each determination). Staining in untreated cells was less intense compared to okadaic acid-treated cells and location of the expressed protein was largely cytoplasmic in both. CMT25 cells expressed moderate levels of CDK1, predominantly localized to the cytoplasm. After okadaic acid treatment, more CMT25 cells were labeled (an increase of approximately 40%). CDK1 expression in untreated CMT27 cells was also cytoplasmic. Okadaic acid treatment elicited an increase of approximately 30% in the number of cells with enhanced CDK1 expression compared to untreated cells (Fig. 4A). Enhanced nuclear labeling was also observed in CMT27 cells in which enhanced cytoplasmic CDK1 was evident. Labeling of CMT cells in which an irrelevant isotype control primary antibody was substituted was undetectable (data not shown).

Immunofluorescent antibody labeling provided enhanced resolution regarding spatial



	CMT9	CMT12	CMT25	CMT27	CMT28	CMT47
Transformed Cell Phenotype	Epithelial	Epithelial	Epithelial	Epithelial	Epithelial	Myoepithelial
Cell Cycle Phase-Specific Arrest	S	S	S & G2/M	-	G2/M	-
Percent Apoptotic Cells						
Control	9.0	18.8	4.4	6.6	5.2	10.7
OA Treated	44.4	41.7	47.9	17.9	69.3	10.4

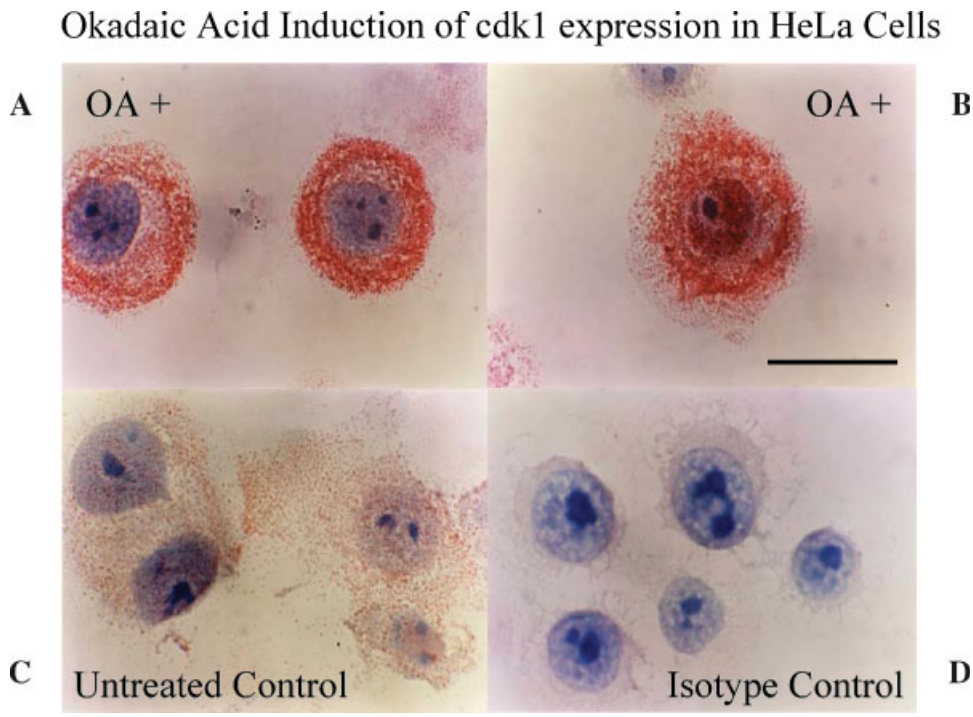


Fig. 3. CDK1 expression in HeLa S3 cells following treatment with okadaic acid. HeLa cells were treated for 24 h with 19 nM okadaic acid, fixed, and labeled with anti-CDK1 monoclonal antibody. Secondary rabbit anti-mouse antibody conjugated to biotin was then applied followed by streptavidin conjugated-horseradish peroxidase and then enzyme substrate and the cells counterstained with H&E. Intensity of the red precipitate indicates the approximate level of CDK1 protein present. **A**: Okadaic acid treated HeLa cells exhibiting enhanced cytoplasmic labeling. **B**: Okadaic acid treated HeLa cells exhibiting enhanced cytoplasmic and nuclear labeling. **C**: Untreated control HeLa cells. **D**: Untreated control HeLa cells in which an irrelevant isotype control primary antibody was substituted. Bar is 20 μm.

distribution of CDK1 that was difficult to observe with immunohistochemistry. Immunofluorescence was used to investigate CDK1 protein expression and localization in the three remaining CMT cell lines (Fig. 4B). Untreated CMT12, CMT28, and CMT47 cell lines expressed uniform cytoplasmic CDK1 expression levels and a minority (not more than 5%) of cells in each line contained detectable nuclear CDK1. Following okadaic acid treatment, the majority of CMT12, CMT28, and CMT47 cells (95% or more) were labeled at similar levels in the cytoplasm, suggesting little change in the amount of CDK1 protein present in most cells. Nuclear labeling appeared unchanged or slightly enhanced in all three CMT cell lines following okadaic acid treatment with the exception that distinct granular foci were apparent in some okadaic acid-treated cells (5–25% of cells in each line exhibited nuclear CDK1 foci). Labeling of CMT cells in which an irrelevant isotype control primary antibody was substituted was undetectable (data not shown).

Accumulation of CDK1 in the Nucleus Is Focal and Involves the Nucleolus

Confocal microscopy was performed to better resolve CDK1 labeled nuclear foci and to determine if CDK1 was actually present in the CMT cell nuclei following okadaic acid treatment. Confocal microscopy was also employed to confirm that apparent nuclear labeling was not

Fig. 2. Cell cycle phase distribution of CMT cells treated with okadaic acid. CMT cells representing six independent cell lines (of epithelial or myoepithial cell origin) were evaluated for DNA content by PI staining after treatment with 19 nM okadaic acid for 48 h. Treated and untreated cell cultures were harvested by trypsin digestion, fixed, and stained with PI and analyzed by flow cytometry. Approximately 10,000 events were counted for each sample and frequency (cell number) was plotted as a histogram against fluorescence (DNA content). Populations of cells in treated versus untreated cultures representing each cell cycle phase were identified and anomylous peaks in treated (arrested) populations noted. The percentage sub-G1 phase (sub-2N DNA content) cells within the population was gated and quantified for each population and the fold increase in apoptotic cells calculated for treated versus untreated control cell populations.

due to superimposed cytoplasmic labeling above or below nuclei. Three-dimensional confocal microscopy of okadaic acid-treated CMT28 and CMT47 cells revealed intense labeling around the nuclear envelope as well as the presence of apparent nuclear CDK1-labeled foci that were intensely labeled in both cell lines (Fig. 5). To confirm the location of the CDK1-labeled foci, a series of 2 μm confocal sections revealed clearly that the areas of bright punctate focal labeling resided within central nuclear sections and not in overlying or underlying cytoplasmic layers. These results were evident in examples of compiled 3-dimentional views (CMT47) as well as individual central nuclear sections (CMT28) from four of the cell lines (representative examples from two cell lines are shown) to resolve CDK1 containing foci within the nuclei.

Additional analysis also suggested that at least some of the nuclear CDK1 foci may be localized within the nucleoli following okadaic acid treatment. To better resolve this question, CMT28 cells were dual labeled with antibodies recognizing CDK1 and nucleolin (Fig. 5B). Although not easily recognizable in control untreated cells, clear indications of coincident staining of nuclear CDK1 foci (green Alexa Fluor 488 fluorescence) within nucleoli stained for nucleolin (red Alexa Fluor 594 fluorescence) as apparent yellow foci (merged green fluorescence coincident with red fluorescence) in the merged images were apparent in okadaic acid

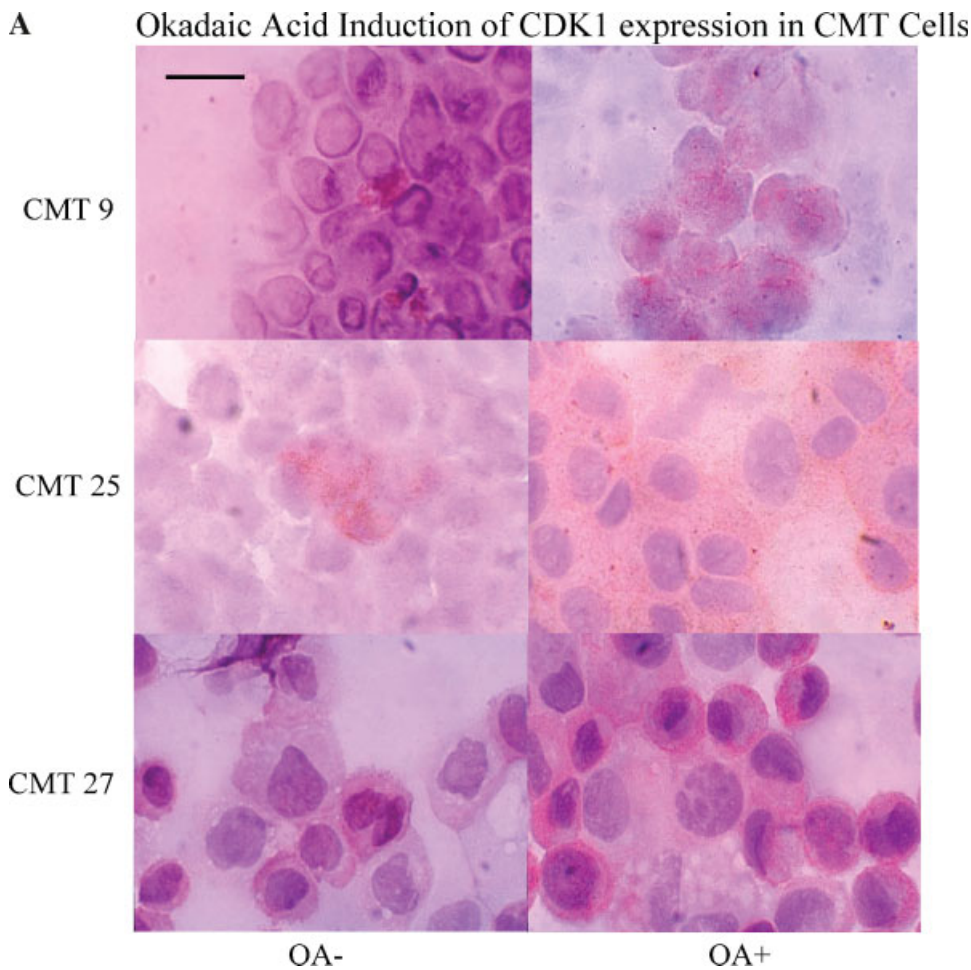


Fig. 4. Immunohistochemical and immunofluorescent labeling of CDK1 protein in okadaic acid treated CMT cell lines. **A**: Immunohistochemical labeling of CDK1 protein expression in three CMT cell lines evaluated following treatment with 19 nM okadaic acid for 24 h, fixation and labeling with anti-CDK1 monoclonal antibody, and counterstained with H&E. Bar is 20 μm. **B**: Immunofluorescent labeling for CDK1 protein expression of three CMT cell lines evaluated following treatment with 19 nM okadaic acid for 24 h, fixation and labeling with anti-CDK1 monoclonal antibody. Arrows note the location of nuclear inclusions/foci brightly labeling for CDK1. Bar is 25 μm.

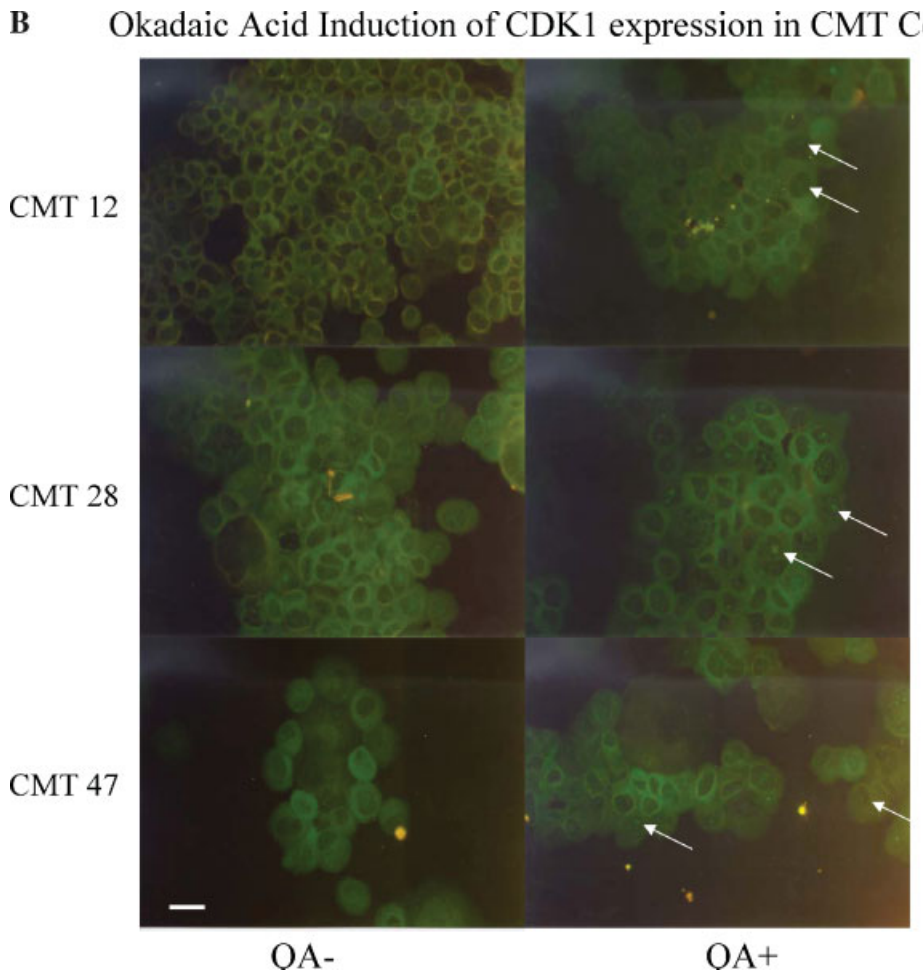
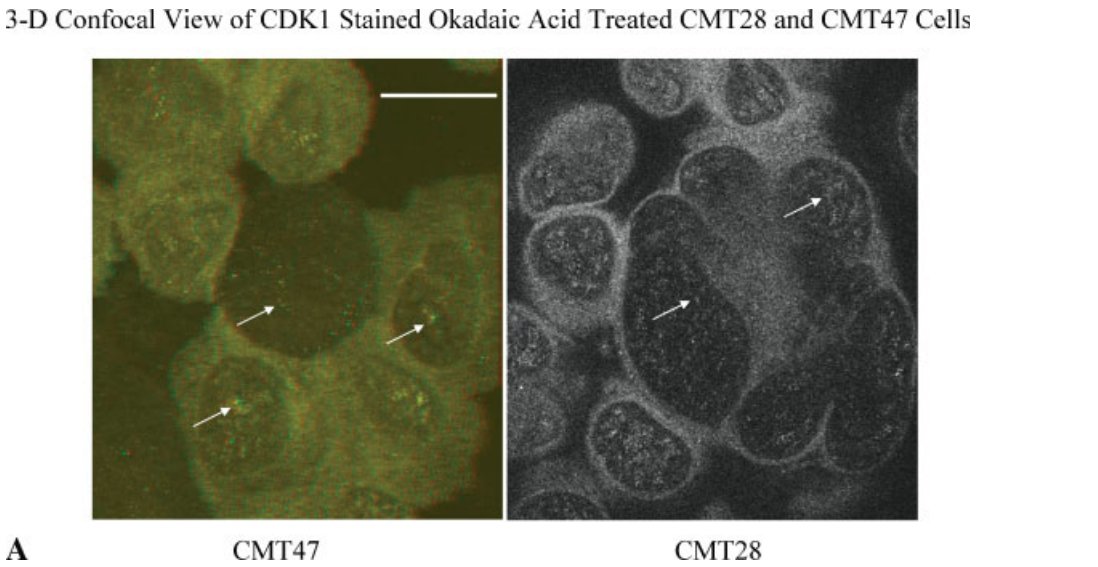


Fig. 4. (*Continued*)

treated cells. Nucleolar CDK1 foci were more abundant and more intensely stained in okadaic acid treated cells although abundant nuclear CDK1 foci were labeled throughout treated cell nuclei outside the nucleolar regions as well.

DISCUSSION

We previously characterized induction of CDK1 transcription by okadaic acid as a model of early events in the G0/G1 phase transition. This analysis allowed identification of an okadaic acid response element (OARE) in the human CDK1 promoter in HeLa cells that appeared to bind a CDP-like factor [You and Bird, 1995; Liu and Bird, 1998; Bird and DeInnocentes, 2004]. However, considerable variability in response to okadaic acid has been previously described, from NIH 3T3 cells which exhibit no stimulatory effects on CDK1 expression after okadaic acid treatment, to the greater than 10-fold levels of CDK1 induction exhibited by HeLa cells [Schonthal and Feramisco, 1993; You and Bird, 1995; Liu and Bird, 1998; Bird and DeInnocentes, 2004]. Because regulation of cell cycle progression is so well conserved and controlled, we hypothesized that the most likely explanation for such different responses may be found in the characteristics of different cell/tumor types or the specific mutations responsible for transformation/immortality in different cell lines. To test this hypothesis, six CMT-derived cell lines with well-characterized biological properties [Wolfe et al., 1986] were evaluated for CDK1 expression and phase of cell cycle arrest in response to okadaic acid treatment. Five of the CMT cell lines were derived from spontaneous epithelial cell tumors and a sixth (CMT47) was derived from a spontaneous myoepithelial cell tumor [Wolfe et al., 1986]. Although these cell lines are all immortal and transformed, they were all independently



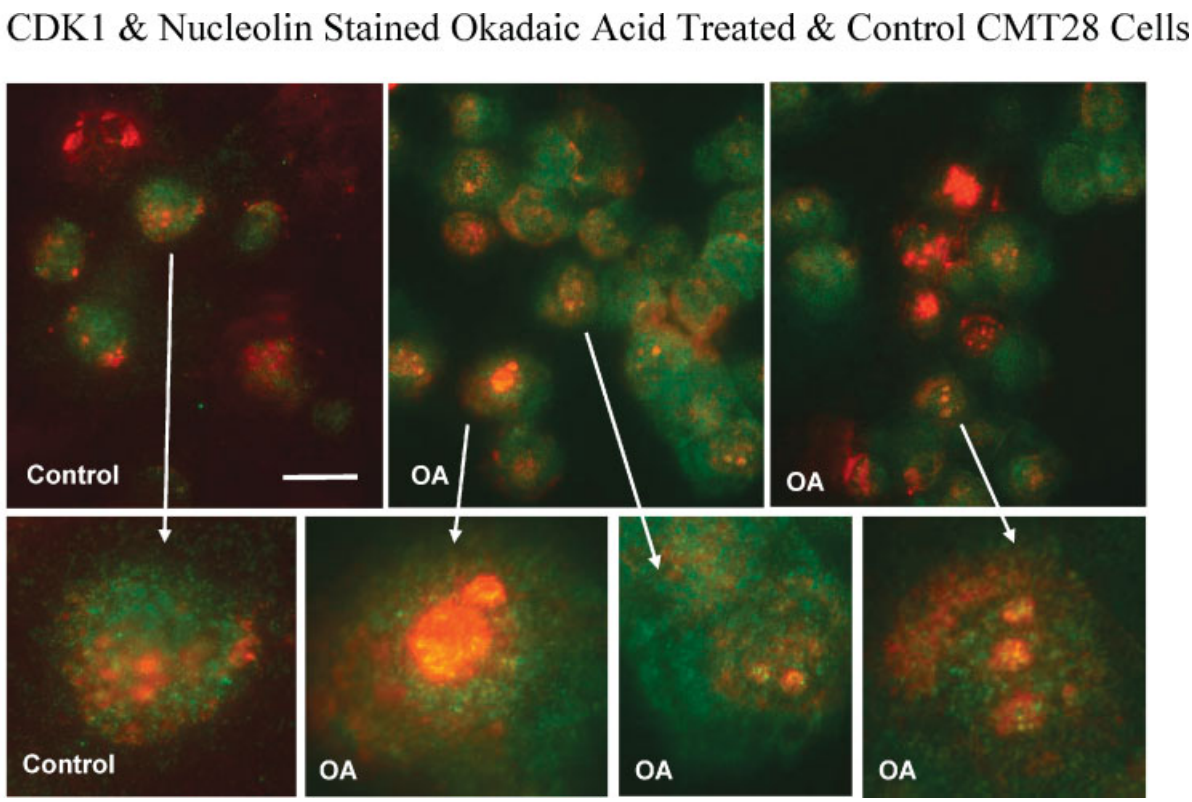


Fig. 5. Confocal and high resolution immunofluorescent microscopy of nuclear and nucleolar CDK1 foci in okadaic acid treated CMT28 and CMT47 cells expressing CDK1. **A**: CMT28 and CMT47 cells were treated with okadaic acid, fixed and immunofluorescently labeled with FITC as described for Figure 4 and confocal micrographs prepared. Arrows note the location of nuclear inclusions/foci brightly labeling for CDK1. **Left**: Three-dimentioanl color offset view of CMT47 cells. **Right**: Central nuclear 2 μm section through similarly treated CMT28 cells demonstrating the presence of CDK1 labeled complexes. Bar is 20 μm. **B**: CMT28 cells were indirectly dual-labeled with antibodies recognizing CDK1 (green Alexa Fluor 488 fluorescence) and nucleolin (red Alexa Fluor 594 fluorescence) following treatment as described above. Coincident staining of nuclear CDK1 foci (green fluorescence), within nucleoli stained for nucleolin (red fluorescence), are apparent as yellow CDK1 foci within the red nucleoli in the merged images. Higher magnification merged images of selected nuclei (origins identified by arrows) are shown (100× oil immersion). Bar is 25 μm.

derived from different animals and thus likely represent a range of different transforming mutations. Characterization of CDK1 regulation in these cell lines allowed evaluation of whether such differences play a role in the reaction to okadaic acid, and possibly in the G0/G1 phase transition.

In contrast to the induction of CDK1 mRNA in HeLa cells from barely detectable levels to levels more than 10-fold higher [You and Bird, 1995; Liu and Bird, 1998], our results demonstrated more heterogeneity in CDK1 mRNA levels in response to okadaic acid treatment among different CMT cell lines. CDK1 mRNA levels ranged from barely detectable to abundant in untreated cultures of the six CMT cell lines. Similarly, CMT cell responses to okadaic acid were very heterogeneous, ranging from little change, to several fold enhancement and even suppression of CDK1 expression in one CMT cell line. The most likely explanation is that these responses are the result of heterogeneity in the genotype/defects, affecting cell cycle regulation, present in individual CMT cell lines.

Immunohistochemical labeling of HeLa cells clearly confirmed enhanced CDK1 protein levels due to okadaic acid, coordinate with induction of CDK1 mRNA, and that treatment resulted in expression of abundant cytoplasmic, and in some cells, nuclear CDK1. In contrast, immunohistochemical and immunofluorescent labeling of CDK1 protein in CMT cells revealed changes in the subcellular distribution and location although at levels of enhancement of expression that were more variable and lower than HeLa cells. Following okadaic acid treatment, cytoplasmic expression ranged from little detectable change (CMT28 and CMT47) to enhancement of CDK1 protein levels of up to several fold (CMT12, CMT9, CMT25, CMT27) over control cytoplasmic levels in at least a portion of the population. These effects were not coordinate with either the range of changes or the differences observed at the level of CDK1 mRNA. In five of six cell lines (all except CMT25), okadaic acid treatment also caused enhanced localization of CDK1 within CMT cell nuclei with evidence of more intense punctuate nuclear CDK1 foci in non-mitotic cells. This was an unusual observation because CDK1 has been thought only to become localized to the nucleus at the onset of mitosis [Takizawa and Morgan, 2000]. It has also not been previously observed to associate in clusters to our knowledge.

Confocal microscopy was used to provide higher magnification regarding CDK1 expression and enhanced analysis of spatial localization, including 3-dimensional image constructions and improved contrast. Okadaic acid-treated CMT28 and CMT47 cells expressed little change in the level of cytoplasmic expression of CDK1 protein levels; however, more intense perinuclear localization was revealed in treated cells using this approach. Bright punctate nuclear labeling was also observed confirming the presence of intensely labeled foci of CDK1 protein in multiple nuclear locations within non-mitotic cells. The nature of the nuclear CDK1 foci could not be identified; however, they formed intriguing structures that occurred in varying density levels and sometimes appeared in clusters. Both single and clustered foci were clearly visible within nuclei and many CDK1 foci also appeared to be localized within the nucleoli though not exclusively in this location. We speculate that these structures represent a local nuclear overabundance of CDK1 complexes within nuclei outside the traditional phase of G2/mitosis as they were present in a majority of interphase cells. We also appear to have evidence of a unique association between CDK1 and the nucleolus that was only revealed due to the overabundance of CDK1 following treatment. CDK1 has recently been implicated in the regulation of the mitotic exit activator cdc14 in budding yeast [Azzam et al., 2004]. Regulation is proposed to involve direct phosphorylation of Net1, part of the Cdc14 early anaphase release (FEAR) network, by CDK1/cyclin B complexes effecting release of Cdc14 from the nucleolus and mitotic exit. The nucleolar complexes visualized in the present study may represent such complexes induced to form during interphase by okadaic acid induced perturbances in the signal transduction system.

These results suggest that although CMT cell CDK1 mRNA levels were heterogeneously affected by okadaic acid, levels of CDK1 protein were modulated to reduce this heterogeneity. Thus, highly variable levels of CDK1 mRNA were modulated to more moderate CDK1 protein expression levels that were less than proportional to the variations observed in CDK1 mRNA levels. Because few of the effects on CDK1 mRNA were observed at the level of CDK1 protein, the implication is that modulation of CDK1 translation, or possibly degradation, appeared to compensate for much of the

variation observed in the level of CDK1 mRNA. It is unknown whether okadaic acid also affects the kinase activity of CDK1 in CMT cells although the evidence from other systems provides conflicting interpretations. At least in neuronal cells in brain, CDK1 kinase activity is unaffected by okadaic acid treatment [Bennecib et al., 2000]. In contrast, clear activation of existing CDK1 kinase activity by okadaic acid has been demonstrated in mouse embryos and in *Xenopus* oocyte extracts suggesting modulation of CDK1 kinase activity by okadaic acid is at least possible [de Vant'ery et al., 1996; Karaiskou et al., 1998]. Despite modulation of CDK1 protein expression in CMT cells, clear effects of okadaic acid on cell cycle progression in CMT cells were still observed. Okadaic acid treatment resulted in perturbations in cell cycle progression that manifested themselves as arrest in S or G2/M phases of the cell cycle or large increases in the apoptotic cell population in all CMT lines of epithelial origin. CMT47 cells were the only exception in that no difference in percent apoptotic cells or cell cycle phase-specific arrest were observed although the cells did arrest (data not shown). CMT47 cells were the only cell line of myoepithelial origin in this study and these differences may stem from this distinct cellular origin.

We conclude that highly variable CDK1 mRNA levels alone, even modulated at the level of translation, do not provide a consistent explanation for the largely consistent pattern of cell cycle arrest and apoptosis except that all cells arrested cell cycle progression. Although okadaic acid stimulation has been shown to mimic many of the early proliferation events observed as cells re-enter the cell cycle, the observation that regulatory responses and characteristics vary between cell lines for mammary tumors of epithelial and myoepithelial origin suggests that cell cycle arrest is due to more than the presence of excess CDK1. Secondarily however, it is clear that regulation of CDK1 is altered as it becomes localized in focal complexes in the nuclei, including the nucleoli, of okadaic acid-treated non-mitotic CMT cells arrested in the cell cycle. Enhanced CDK1 expression appears to be only part of the explanation for okadaic acid-induced cell cycle arrest and it is possible that displacement of CDK1 to the nucleus and/or the nucleolus during interphase is actually the deregulating event. Such displacement may not require CDK1 overexpression and may be sufficient to arrest cell cycle progression. CDK1 suppression of other CDK enzyme activities, normally expressed in other phases of the cell cycle, provides a possible mechanism that explains how nuclear CDK1 outside mitosis could arrest cell cycle. The presence of excess CDK1 in the nucleus, outside G2/mitosis, could suppress other CDK activities normally required to progress into S and G2/M phases where four of the cell lines arrested [Bird, 2003]. Redistribution of CDK1 protein, within the cytoplasm and into the perinuclear region, also supports such a model as it suggests an increase in the nuclear affinity of CDK1. Cyclins contain nuclear localization information not found in CDKs and it is these signals that appear to promote nuclear importation of CDK/cyclin complexes as cells approach division [Devault et al., 1991; Parker and Piwnica-Worms, 1992; Van den Heuvel and Harlow, 1993]. The demonstration that control of cyclin/CDK localization is dependent on phosphorylation is also consistent with the deregulating effects of phosphatase inhibition by okadaic acid [Yang and Kornbluth, 1999]. Cell cycle arrest following okadaic acid treatment may be the result of such deregulating effects on these cyclin/CDK1 localization mechanisms in conjunction with induction of CDK1 expression. CDK1 suppression of inappropriate CDK activation outside the mitotic phase may be more complex and important than previously thought and a key regulator of cell cycle arrest.

Deregulation of proteins controlling cell cycle progression frequently leads to cell transformation and this has provided a strong rationale for identification of targets among these genes that can be manipulated to arrest proliferating cancer cells or induce apoptotic death. Because there is little evidence that CDK1 can behave as an oncogene, CDK1 is particularly well suited as such a target as CDK1 expression is required for proliferation and overexpression induces apoptosis [Hsu et al., 1999]. Manipulation of CDK1 levels can thus be proposed as a potential target for genetic suppression strategies designed to arrest cell cycle or induction strategies designed to induce apoptosis with a lower predicted level of oncogenic risk than other gene targets. Although cell cycle arrest is a common consequence of manipulation of CDK1 levels, this study demonstrates the importance of understanding the complexity and hetero-

geneity of cell cycle regulatory mechanisms to best exploit their value as targets for antiproliferative strategies in design of anticancer therapeutics.

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