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mRNA Expression Profile of a Human Cancer Cell Line in Response to *Ginkgo Biloba* Extract: Induction of Antioxidant Response and the Golgi System

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Supplementation of diets with plant extracts for health and prevention of degenerative diseases is popular. However the molecular basis of their therapeutic potentials are poorly defined. We hypothesized that *in vitro* assays that enable quantitative analysis of the gene expression profiles combined with targeted biochemical analysis can identify the potential effects of phytochemicals. The hypothesis was tested by application of GeneChips to define mRNA expressions of a human bladder cancer cell line incubated with a flavonoid containing extract of *Ginkgo biloba* leaves. The analysis of the transcriptional response revealed a net activation of transcription. Functional classification of the affected mRNAs showed the largest changes in the abundance of mRNAs for intracellular vesicular transport, mitochondria, transcription and antioxidants. The transcripts for hemeoxygenase-1, mitochondrial superoxide dismutase and the regulatory subunit of γ-glutamyl-cysteinyl synthetase and their encoded proteins were elevated. The extract also increased intracellular glutathione, the transcripts for DNA repair and synthesis, and decreased ^{3}H-thymidine incorporation. These results demonstrate that a flavonoid containing extract initiates an adaptive transcriptional response that augments the "antioxidant status" of the cells and inhibits DNA damage. These *in vitro* studies using GeneChips demonstrated a promising strategy for identifying nutritional supplement induced cellular responses that may have a role in counteracting chronic human diseases.

Keywords: activated ras, reactive oxygen species, oligonucleotide arrays, dietary antioxidants

Abbreviations: SOD, superoxide dismutase; DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction

INTRODUCTION

It has been argued that metabolically generated oxidant by-products are major contributors to chronic disorders such as coronary heart disease and cancer [1]. Dietary supplements with antioxidant properties such as *Ginkgo biloba* extracts, vitamin E, and carotenoids have, therefore, enjoyed popular use as remedies for chronic disorders. The increased practice of supplementation of diets with phytochemical extracts is also fueled by numerous epidemiological studies that have shown that dietary consumption of fruits

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and vegetables, which also have antioxidant properties, decreases the risk of coronary heart disease and cancer [2,3]. Such well established epidemiological data have stimulated research into the genetic manipulation of plants to augment the production of bio-active phytochemicals such as vitamin E [4,5] and provitamin A [6].

Although epidemiological studies have identified the intake of natural dietary components with decreased incidence of complex pathologies, the causative molecular actions of such complex plant mixtures remain essentially elusive and difficult to define. Quantitative and comprehensive *in vitro* assays that can screen the potential of an extract to affect cellular functions are essential to identify the mechanisms underlying the potential therapeutic actions of plant extracts. Recent developments of techniques for simultaneous and quantitative analysis of the expression of large numbers of mRNAs [7, 8] and proteins [9] has offered powerful tools to interrogate the responses of an entire genome of a cell [10] or even a multicellular organism [11]to physiological and environmental stimuli. The drug discovery program conducted by Developmental Therapeutics Program of National Cancer Institute (NCI) to define molecular actions of synthetic compounds and natural products illustrates the potential of this analytical strategy. In addition to the application of classical assays of cytotoxicity and growth inhibition, the more recently invented assays of global gene expression profiling identified the activities of 70000 different compounds, including natural products, in 60 different cancer cell lines [12,13].

Human bladder cancer cell line (T-24) with constitutively active, oncogenic G-protein, Ha-ras [14], offers an attractive *in vitro* model to test the transcriptional response of a metabolically active cell to a selected plant extract with antioxidant properties. It has previously been shown that high expression of Ras, a member of G-protein superfamily essential in the signal transduction pathways from the membrane to the nucleus, increases reactive oxygen species and cellular proliferation, both of which were inhibited by antioxidants such as N-acetyl cysteine or superoxide dismutase [15]. An extract of *Ginkgo biloba* leaves has been shown to have antioxidant properties attributed primarily to the presence of flavonoid-glycosides in the extract. The antioxidant effects of flavonoids are achieved by either directly scavenging reactive radicals [16]or by chelating, prooxidant, transition metal ions [17]. Since reactive oxygen species regulate the expression of a large number of genes [18] we proposed that the evaluation of the gene expression profile of T-24 cells in response to exposure to an antioxidant extract should identify genes sensitive to intracellular reactive oxygen species.

Flavonoids may also affect cellular functions and may target transcription processes independent of their antioxidant properties. Flavonoids that inhibit cyclin dependent kinases and topoisomerase-I have been developed to control cellular proliferation, differentiation and apoptosis [19–21]. For example, flavopiridol, a synthetic flavone has been shown to be a potent inhibitor of protein kinases and it inhibits the growth of several cancer cell lines [22]. Other pharmacologically active components in extracts of *Ginkgo biloba* leaves are terpenoids [23]. The biological effects of plant derived terpenoids on mammalian cells are less well defined, but they have been shown to arrest cells in the G1 phase and initiate apoptosis by inhibition of mevalonate synthesis, which is required for prenylation of ras and lamin B[24].

In this study high density oligonucleotuide arrays were applied to define the potential of the extract of *Ginkgo biloba* leaves, EGb 761, to affect the transcriptional response of a human cancer cell line. The extract sensitive gene expression data obtained by this analytical strategy may prove useful in evaluating the potential therapeutic claims of complex extracts and in designing objective *in vivo* studies.

METHODS

Human Bladder Cancer Cell Line (T-24) Cell Line

The cell-line was obtained from the American Type Culture Collection, Rockville, MD. The cell line (T-24) was propagated in Medium 199 with fetal calf serum (10%), streptomycin (100μg/ml) and penicillin (100μg/ml), at 37°C with 5% CO_2 and air. The cells used in this study had undergone 9 to 15 passages in culture. The HaCaT cell line, a non-tumoral human keratinocytes cell line[25], was provided by Professor N. E. Fusenig (German Cancer Research Center, Heidelberg, Germany). The cells were grown in a Dulbecco's Modified Eagle Medium (GIBCOBRL) with low glucose supplemented with 10% (v/v) heat-inactivated fetal calf serum with streptomycin (100μg/ml) and penicillin (100μg/ml), in a humidified atmosphere with 5% (v/v) CO_2 at 37 °C.

Incubation of Cells with *Ginkgo Biloba* Extract, EGb 761

Dried *Ginkgo biloba* extract (a gift from Ipsen, Paris, France) was dissolved in dimethyl sulfoxide (DMSO) to obtain a stock solution of 100 mg/ml. Aliquots (e.g. 1μl) of the stock extract solution was added per ml of Medium-199 with (10%) fetal calf serum, streptomycin (100μg/ml) and penicillin (100μg/ml) over confluent cells and then incubated for up to 72h. The concentration of EGb 761 (100ug/ml) selected for the evaluation of the transcriptional response was based on numerous *in vitro* studies including those showing inhibition of protein phosphorylation in human vascular endothelial cells, oxidation of apolipoprotein E and neuronal death induced by oxidative stress [23]. A sample of *Ginkgo biloba* extract was submitted to the National Cancer Institute (NCI), for evaluation as an antiproliferative agent against a panel of 60 human cancer cell lines [26].

Preparation of Target RNA

Biotinylated complimentary RNA fragments were prepared for hybridization to the oligonucleotide probes of the Hu6800 GeneChips as described in the Expression Analysis Technical Manual, (Affymetrix, Santa Clara, CA). Briefly, cDNA was prepared from mRNA (~5μg) with the T7 specific oligonucleotide-$dT_{(24)}$primer. The second strand of DNA was synthesized with DNA polymerase I, DNA ligase and RNAaseH as described in the manual. Double stranded DNA (~2 μg) was used for the synthesis of biotinylated RNA with an *in vitro* transcription kit (Ambion, Austin, TX). Amplified biotinylated-RNA was purified, fragmented, and mixed with internal standards prior to hybridization to the probes on the GeneChips. Following a 16 hour hybridization in a rotisserie oven at 40°C, the chips were washed to remove unhybridized target RNA and stained using a fluorescently labeled streptavidin antibody, then scanned with a Hewlett-Packard GeneChip scanner.

Data Analysis

The GeneChip data from the scanner were analyzed as described in the Affymetrix GeneChip Expression Analysis manual. Positive hybridizations of transcripts from T-24 cells to probes on the GeneChips were identified as "absolute presence" of mRNAs from control cells and from the extract treated (6h, 48h and 72h) cells. In order to determine the effect of the extract at each of the incubation time on each of the transcripts a *Comparison Analysis* was performed after *Normalization and Scaling* of the data obtained at each time point. The transcripts identified by *Difference Call* as either increased or decreased *and* showing two-fold or greater change were selected as candidate mRNAs whose abundance was significantly altered by the extract.

Confirmation of Selected mRNA Transcripts

Total RNA was extracted with Trizol Reagent (GIBCOBRL) from confluent T-24 cells exposed to either DMSO (0.1%) or the extract, EGb 761, as described above. A fraction (50 μg) of total RNA was treated with DNAase (GenHunter Corporation, Nashville TN) to remove genomic DNA. A fraction (1–4 μg) of DNAase treated RNA was reverse transcribed with Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase primed with dT_{15} primer according to manufacturer's protocol (GIBCOBRL).

Aliquots of diluted cDNA were used as templates for amplification of specific fragments of selected transcripts by polymerase chain reaction (PCR) with Taq polymerase (GIBCOBRL) in the presence of transcript specific primers. The PCR primers for specific transcripts were as follows: heme-oxygenase-1 [27]: sense, gaattcagcatgccccaggatttg, antisense, tctagactagctggatgttgagcagga; galectin-1 [28]: sense, tggactcaatcatggcttgtgg, antisense, ctggctgatttcagtcaaagg; ribosomal L37, sense, aagcgagatgacgaagggaacg, antisense, tagccacatttgccacaggtcg, human ribosomal protein L3, sense cagcagacaggcatacagaggttc, antisense, tactgacaggaggcaggtgaatgg. The primers were custom synthesized by Operon Technology Inc., Alameda, CA or GIBCOBRL, www.lifetech.com. The primers for human GAPDH, and β-actin were obtained from Stratagene (La Jolla, CA).

The polymerase chain reaction solution contained Tris-HCl (50mM, pH 8.4), KCl (50 mM), dATP (0.2 mM), dCTP (0.2 mM), dGTP (0.2 mM), dTTP (0.2 mM), $MgCl_2$ (1.5 mM), primers (0.5 μM each), cDNA template (<0.1 μg), Taq DNA polymerase (2.5 units) in a volume of 20 μl. The PCR amplification cycles were as follows: 94°C for 45 sec, 60°C for 45 sec, 72°C for 1.5 min, for 35 cycles followed by 10 min at 72°C and cooled to 4°C. The PCR products were resolved on 2% agarose gel in Tris (0.1M)-boric acid (0.09M)-EDTA (0.001M), pH8, with ethidium bromide and visualized under UV. Polaroid photographs were obtained and analyzed with NIH Image analysis software.

^{3}H-Thymidine Incorporation

The cells were seeded at a density of 2–4 × 10^4/ml, 3ml/well and grown to confluence in Falcon 12 well, flat bottom plates (Becton Dickinson, NJ). Cells treated with *Ginkgo biloba* (100 μg/ml) or with DMSO (0.1%), for the specified times were incubated with 1 μCi/well of ^{3}H-thymidine (NEN Life Science Products, Boston MA) for 4 hours. The cells were harvested and assayed for ^{3}H-thymidine incorporation into trichloroacetic acid (5%) insoluble pellets. This is a measure of thymidine incorporation into cellular DNA.

Evaluation of Protein by Immunoblot (Western) Analysis

The cells were grown and treated with the extract as described above. An aliquot (250 μl/well) of lysis buffer containing protease inhibitors (SantaCruz Biotechnology, CA) was added and the samples were stored at –80°C until ready for analysis by denaturing polyacrylamide gel electrophoresis. The cellular proteins were resolved on precast, gradient (5–20%) minigels and transferred to nitrocellulose membranes as described in Novex (San Diego, CA) protocols. Antisera for human heme-oxygenase-1 and human Mn-SOD were obtained from StressGen Biotechnologies Corporation (Victoria, B.C.) and used as described by the manufacturer to detect the immunoreactive proteins immobilized on nitrocellulose membranes. The antisera to γ-glutamyl-cysteinyl synthetase regulatory and catalytic subunits was a gift and we thank Drs, T Kavanaugh, C. Kejsa (University of Washington.), G. Schieven (Bristol Meyers Squibb) and C. Saliou (U.C. Berkeley).

Glutathione and Peroxide Levels

The metabolites were measured in confluent cells exposed to the extract of *Ginkgo biloba* or with DMSO as described above. Reduced glutathione was detected using an HPLC coupled to an electrochemical detector as described previously [29]. To determine cellular peroxide levels, cells were incubated with 2′,7′-dichlorofluorescein-diacetate for 60 minutes and then washed with ice cold phosphate buffered saline. Intracellular oxidized fluorescent 2′,7′-dichlorofluorescein was measured using a fluorescense microplate reader.

Flow Cytometry, Cell Cycle and Cell Proliferation Analysis

The cells were grown in 12 well flat bottom plates as described above. After incubation of the cells with the extract the cells were harvested by incubation with trypsin to detach the cells from culture plate, washed twice with phosphate buffered saline and prepared and analysed as described in[30]. Cell proliferation was assayed by Celltiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, USA).

RESULTS

The presence of flavonoid glycosides in the extract of *Ginkgo biloba*leaves and the extensive biochemical literature on the antioxidant properties of flavonoids and their effects on signal transduction pathways, cell differentiation, proliferation and apoptosis offer compelling reasons to the determine the extract sensitive mRNA expression profile of a malignant cell. Human transitional bladder cancer cells (T-24) were incubated with *Ginkgo biloba* extract, EGb 761 for up to 72 hours. Analysis of the mRNA transcripts of control cells (exposed to vehicle, DMSO, 0.1%, for 72 hours) revealed hybridization to probes for 1980 distinct mRNAs out of oligonucleotide probes for 6936 mRNAs on Affymetrix Hu6800 GeneChips. This shows that the sensitivity of ~ 2000 genes expressed in T-24 cells could be studied with the oligonucleotide arrays on the GeneChips. The most recent estimate of the number of transcripts in a cancer cell is 43,500 mRNAs [31]. Therefore, in this study only a small fraction (~5%) of the expressed genome of human T-24 cells was screened for its sensitivity to the extract of *Ginkgo biloba* leaves. The experiment revealed a transcriptional response that showed changes in the expression of at least 155 distinct genes; the expression of 139 genes was up-regulated and the abundance of 16 mRNAs was down-regulated.

The malignancy of T-24 cells is attributed to the presence of an activated c-Ha-ras oncogene [14]. Evaluation of hybridization data showed the presence of 23 Ras related transcripts. The expression of the transcript for c-Ha-ras was higher than 65% of the all ras family of transcripts (Fig 1A). The transcript for ras related GTP binding protein was the highest expressing mRNA of this Ras family and the putative polypeptide encoded by the mRNA contains putative repeats of the Ras-related GTP-binding region [32].

The analysis of T-24 mRNAs identified several transcripts, in addition to Ha-ras that are associated with malignant cell growth. The abundance of these transcripts was either similar to or higher than those of house keeping genes, GAPDH and β-actin (Figure 1B). These mRNAs included transcripts for Wilm's Tumor related protein [33], several ribosomal proteins including L5, L37 [34,35], and S27 [36], the calcium binding proteins, lipocortin and calcyclin; v-fos transformation effector protein; a tumor associated 120kDa nuclear protein; pancreatic tumor related protein and a galectin-like protein [28,37]. The latter had previously been shown to be highly abundant in most high grade tumors of bladder. High abundance of galectin-1, and ribosomal protein L37 mRNAs were confirmed by RT-PCR using transcript specific primers and cDNAs prepared from T-24 cells as shown in

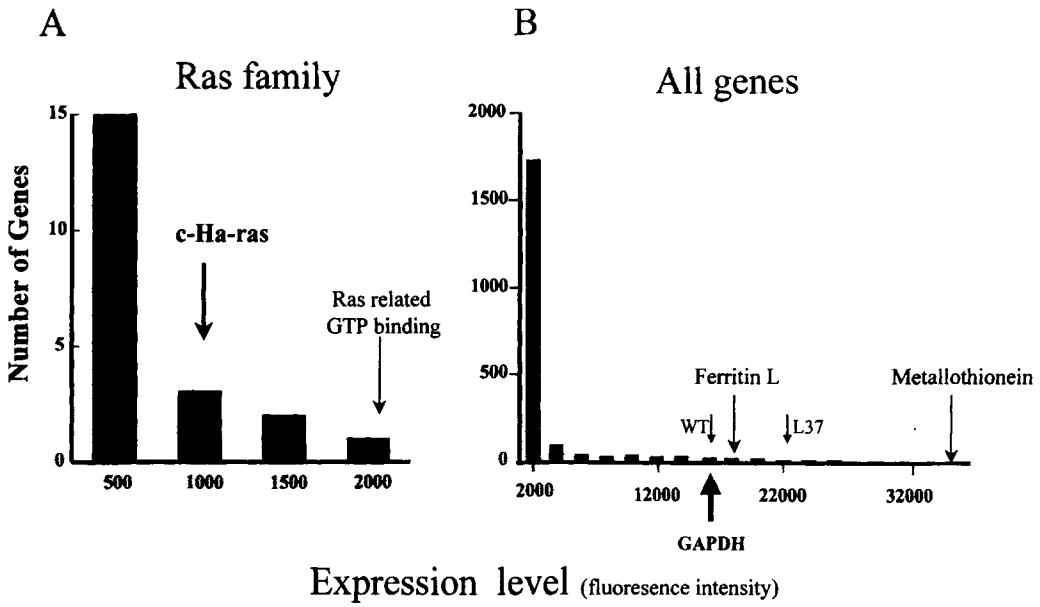

FIGURE 1 mRNAs for c-Ha-ras and metallothinein show high abundance in human bladder cancer cells (T-24). mRNAs identified by GeneChips in T-24 cells under basal conditions were grouped by their abundance as determined by the hybridization signals (fluorescence intensity). A. Twenty-three members of the Ras superfamily were detected; the transcript for c-Ha-ras was expressed two-fold higher than 65% of other Ras transcripts. B. Most of the transcripts (~ 80 % of the total) were expressed at low abundance. mRNA encoding metallothinein showed the highest abundance. Other transcripts associated with malignant growth such as transcripts for Wilms Tumor (WT) suppressor protein and ribosomal proteins e.g. L37 were also present in high abundance relative to that of GAPDH

Figure 2. The figure also confirms that the abundance of the two transcripts was unaffected, for up to 72h, in the presence of the extract.

Cells exposed to *Ginkgo biloba* extract for 6, 48 and 72 hours showed hybridization, as defined by *Absolute Presence*, to 2483, 2285 and 2197 probes on the GeneChips, respectively. Of these, 1658 mRNAs were detected at all of the four time points. Figure 3 shows the distribution of the extract sensitive transcripts identified by "*Difference Call*"(see Methods). A complete list of the genes whose expression is affected by the extract is provided as the Supplemental Data. The expression of most (~90%) of the genes was unaffected by the extract as shown in Figure 3b.

The analysis of transcripts of the T-24 cells over the period of 72 hours in the presence of the extract showed a net induction in the expression of 139 distinct genes. The transcripts encoding two transcription factors, Lbx1 and MSX-2 were down regulated over the 72 hour incubation with the extract (Table I). Each of these transcription factors plays an important role in tissue specific gene expression by interacting with homeodomains on DNA. MSX-2 is a repressor of transcription [38] and its down regulation would be expected to turn on transcription. The transcript for a basal histone, H2A.Z, which is associated with increase in the rate of transcription [39] was also induced together with the induction of transcripts encoding ubiquitin carrier protein and ubiquitin conjugating, both of which have been shown to be essential in activating H2B, a yeast histone related to H2A.Z [40].

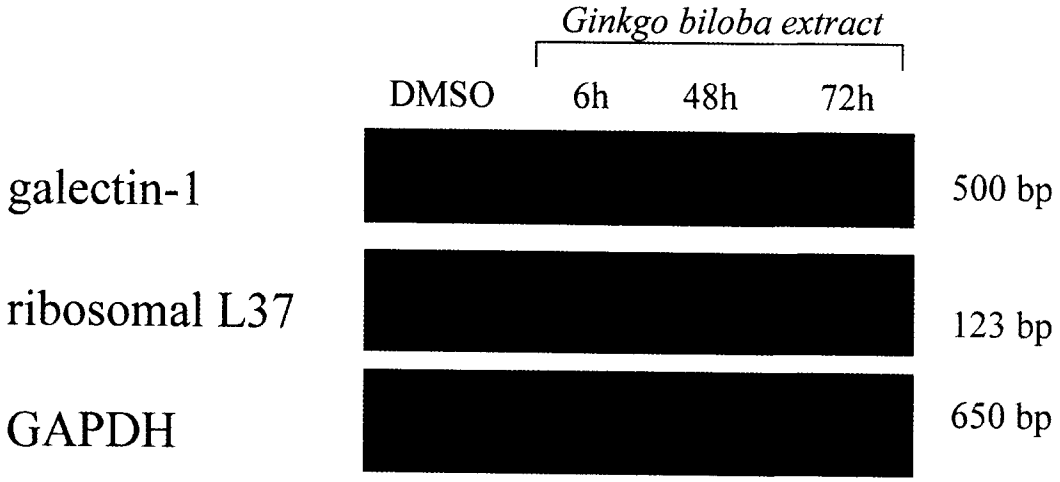

FIGURE 2 Confirmation of high expressing transcripts identified by GeneChips. cDNA templates (diluted identically) from control and the extract treated cells were subjected to PCR analysis with the transcript specific primers as described in the Methods. The transcripts for Galectin-1 and ribosomal protein L37 were present at abundance levels similar to that of GAPDH and their expression was unaffected by the extract, as suggested by the data from GeneChips

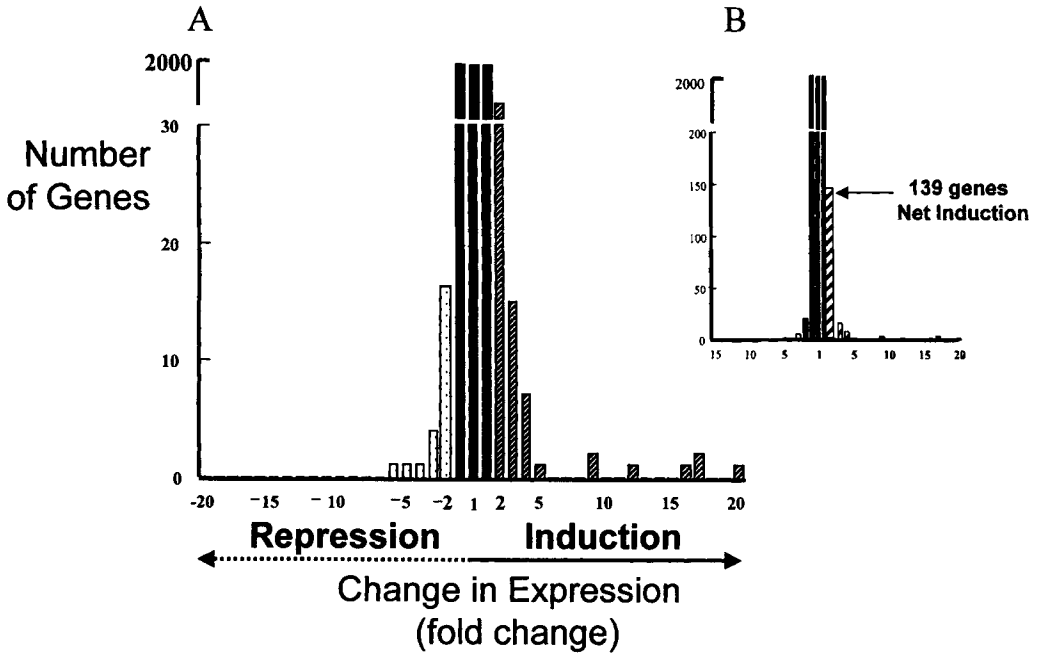

FIGURE 3 Distribution of Ginkgo biloba extract sensitive transcripts in human bladder cancer cells. Most (~93%) of the transcripts identified in control cells were unaffected (determined by "Difference Call" analysis, see Methods) by the presence of the extract over 72 hours of incubation. There was a net induction (ranged from 2 to 20 fold) of 139 transcripts and repression (2–6 fold) of 16 mRNAs compared to the expression of the respective transcripts in control cells

TABLE I Selected list of *Ginkgo biloba* extract sensitive genes encoding transcription factors and proteins for the processing of RNA. The transcripts were identified by "Difference Call" analysis as mRNAs whose expression was affected by two-fold or more in the presence of the extract compared to untreated control cells. The abundance of each transcript in untreated control and in Ginkgo biloba extract treated T-24 cells is shown by fluorescence intensity for each transcript. D = decrease, I = increase

		Fluorescence intensity				
Name of Gene	*Accession #*	*control*	*6 hours*	*48 hours*	*72 hours*	*change*
hypoxia inducible factor	U22431	1211	263	745	746	D
estrogen receptor	S67777	281	456	106	54	D
Lbx1	X90828	768	32	94	120	D
MSX-2	D89377	367	189	106	89	D
global transcription factor	M88163	671	648	915	1638	I
transcription elongation factor	M81601	933	1466	1494	2504	I
pre-mRNA splicing factor	D28423	699	1574	1434	1468	I
NCBP interacting protein	D59253	77	202	107	121	I
H2A.Z	M37583	1889	3295	4145	5509	I
ubiquitin carrier protein	M91670	1375	1306	2406	3605	I
ubiquitin conjugating enzyme,	D83004	228	318	415	583	I
GL1 krupple related protein	M77698	432	608	894	1185	I
CpG associated	S78771	241	429	324	322	I
P3. CpG islands	X12458	97	176	23	340	I
mitochondrial transcription factor	M62810	210	394	591	592	I

Activation of transcription would require simultaneous induction of the RNA processing pathways for the maturation of *de novo* transcribed pre-mRNAs. This was demonstrated by the increase in the expression of transcripts for nuclear cap binding protein, pre-mRNA splicing factor, transcription elongation protein and global transcription activator homologue in the presence of the extract (Table I and Supplemental Data).

Several transcripts encoding proteins with antioxidant functions were induced in the T-24 cells in the presence of the extract as shown in Table II. These included mRNAs for heme oxygenase-1 (HO-1) seen as early as 6h after the addition of the extract, mitochondrial Mn-superoxide dismutase (MnSOD) mRNA and the regulatory subunit of γ-glutamyl-cysteinyl synthetase (GCSr), the rate-controlling enzyme in the synthesis of glutathione. The time course of increase in the abundance of HO-1 transcripts was confirmed by RT-PCR with HO-1 specific primers and cDNA templates prepared from extract treated cells (Figure 4). In addition the increase in the abundance of the three transcripts was translated into increase in the abundance of the respective proteins in cellular extracts of as detected by the specific antisera against the respective proteins (Figure 5). Furthermore, extracted treated cells compared to cells exposed to the vehicle for the three time intervals also showed increased levels of glutathione (Figure 6). Transcriptional induction of HO-1 and GCSr have previously been shown to be regulated by multiple factors, including increase in intracellular peroxides [41,42]. Figure 7 shows the effect of *Ginkgo biloba* extract on intracellular peroxides, as measured by fluorescence of dichloro fluorescein, in the T-24 cells. The accumulation of peroxides was induced at 6h and remained elevated at 48 h in the presence of the extract but

not in the cells treated with the vehicle for 6h and 48h. Table II also lists eleven nuclear encoded mitochondrial transcripts induced by *Ginkgo biloba* extract. Most of these transcripts encode proteins of mitochondrial matrix and three transcripts encode proteins of the inner mitochondrial membrane.

There was a coordinated increase in several transcripts (Table III) that encode vesicular proteins suggesting that the *Ginkgo biloba* extract stimulates the function of the Golgi system which plays a central role in intracellular transport of proteins associated with membrane vesicles [43]. The induction of transcripts for intracellular translocation of the vesicles accompanied the induction of mRNAs encoding vesicular proteins. These included mRNAs for kinesin heavy chain, kinesin-related protein and troponin-T. Kinesins are motor proteins associated with membranous compartments [44]. Such synchronized induction of transcripts encoding vesicular proteins may be essential for assembly and modification of intracellular organelles such as peroxisomes and lysosomes as indicated by in increase in the mRNAs for peroxysomal enoyl hydratase and β-hexoseaminidase, respectively (Supplemantal Data).

TABLE II List of *Ginkgo biloba* extract sensitive genes encoding antioxidant enzymes, heat shock and mitochondrial proteins. The transcripts were identified by "Difference Call Analysis" as mRNAs whose expression was affected by two-fold or more in the presence of the extract compared to untreated control cells. The abundance of each transcript in untreated control cells and in Ginkgo biloba extract treated T-24 cells, at the three incubation periods, is shown by fluorescence intensity

Name of Gene	*Accession #*	*control*	*Fluoresence intensity*		
			6 hours	*48 hours*	*72 hours*
Antioxidant / Heat shock					
gamma-glutamyl-cysteinyl synthetase	L35546	203	564	465	376
aldehyde dehydrogenase 6	U07919	812	627	1221	2062
thioredoxin peroxidase	U25182	370	462	648	910
heme oxygenase-1	X06985	969	4676	2805	2052
p23	L24804	370	461	474	473
Lamin B	M34458	156	281	358	336
immunophilin	M88279	1161	1198	1884	3274
NADPH menadione oxidoreductase	J03934	2184	3053	4968	4175
cysteine rich protein	M76378	1225	1212	1361	2324
Mitochondrial Genes					
NADH-ubiquinone reductase	HG3141	525	1298	1488	1239
mitochondrial matrix protein	M22389	3808	5711	5750	8369
aspartate transferase	M22632	1053	1837	2319	3221
cytochrome c oxidase Va	M22760	1464	2072	3009	3433
somatic cytochrome C	M22877	243	725	692	870
cytochrome b5	M22976	427	458	391	797
NADP dependent malic enzyme	M55905	119	210	254	288
single stranded DNA binding protein	M94556	1554	2375	2870	3385
uncoupling protein homologue	U94592	169	472	79	221
Mn SOD	X65965	469	583	800	639

TABLE III *Ginkgo biloba* extract sensitive genes encoding proteins for vesicular transport and the Golgi system. The transcripts were identified by "Difference Call" analysis as mRNAs whose expression was affected by two-fold or more in the presence of the extract compared to untreated control cells. The abundance of each transcript in untreated control T-24 cells and in Ginkgo biloba extract treated cells, at the three incubation periods, is shown by fluorescence intensity

			Fluoresence intensity		
Name of Gene	*Accession #*	*control*	*6 hours*	*48 hours*	*72 hours*
RAB 1 (ras related protein)	HG 3521	91	275	228	294
RAB 2 (GIP binding protein)	M28213	125	277	209	300
Krev-1 (ras related protein)	M22995	333	345	488	900
mp24, transmembrane protein	X92098	214	494	329	250
synaptobrevin like novel gene	X92396	169	515	387	408
synaptophysin like, hSp1	X68194	161	504	160	132
beta adaptin	M34175	2059	2449	2772	5457
CDC42	U02570	975	1008	1272	2526
alpha tubulin	X06956	389	332	665	782
autocrine motility protein	M63175	314	432	538	748
ADP ribosylation factor	M74491	1689	2930	3876	5756
clathrin light chain B, splice 1	HG 2795	234	4162	2917	2222
non-myosin heavy chain	M31013	2086	2630	3645	6791
kinesin realted protein	D14678	77	103	194	111
kinesin heavy chain	X65873	88	201	258	229
ELP-1 (Brefeldrin A phenotype inducing)	M88458	774	2254	2357	2736

The incubation of bladder cancer cells with the extract from leaves of *Ginkgo biloba* induced at least four transcripts encoding proteins essential for the repair and synthesis of DNA (Table IV). These included the transcripts for DNA mismatch repair enzyme, induced as early as 6h and the transcripts for DNA ligase, the regulatory subunit of DNA polymerase and topoisomerase-I. ^{3}H-thymidine incorporation in cellular DNA of T-24 cells incubated with *Ginkgo biloba* extract was decreased by up to 50% at 6 hours and remained low compared to control cells (Figure 8A) through out the incubation period. Metabolic assay for cell proliferation (see methods) showed that the extract had no effect on proliferation of T-24 cells (Figure 8B). This was further supported by flow cytometry analysis of propidium iodide stained cells. The population of cells in the G-1 phase of the cell cycle (~70–80%) was unaltered for up to 72 hours after exposure to *Ginkgo biloba* extract. The cells showed no evidence of cell necrosis or apoptosis (Figure 9). The transcripts for cyclin B, cyclin D3 and cdc2 in the *Ginkgo biloba* extract treated cells were increased compared to untreated cells. Elevated levels of the D-type cyclins are associated with the progression through G1 [39]whereas elevated levels of cyclin B-cdc 2 complex is associated with G2-M phase of the cell cycle [45]. Sustained increase in the expression of these transcripts would maintain the cells in the G-phase and prevent the cells from entering the DNA synthesis phase of the cell cycle. These changes in gene expression may explain the flow cytometry data that show that ~80% of the cells remain in G1 phase.

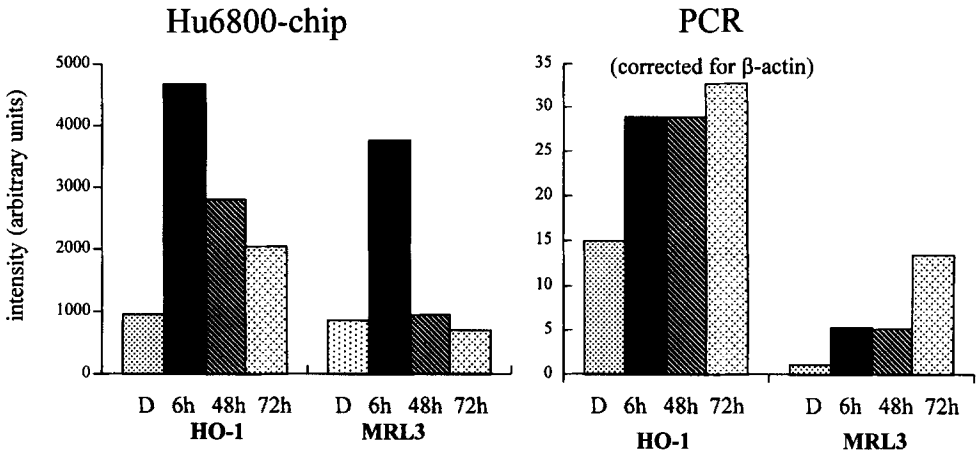

FIGURE 4 Confirmation of GeneChip data by PCR for changes in mRNAs for heme-oxygenase-1 (HO-1) and ribosomal protein, MRL3. Increase in the expression of mRNA for HO-1 in the presence of the extract was detected as early as 6 hours after incubation with the extract and remained elevated during the incubation period. GeneChip data showed an increase in MRL3 after 6 hour and was confirmed by PCR with the transcript specific primers

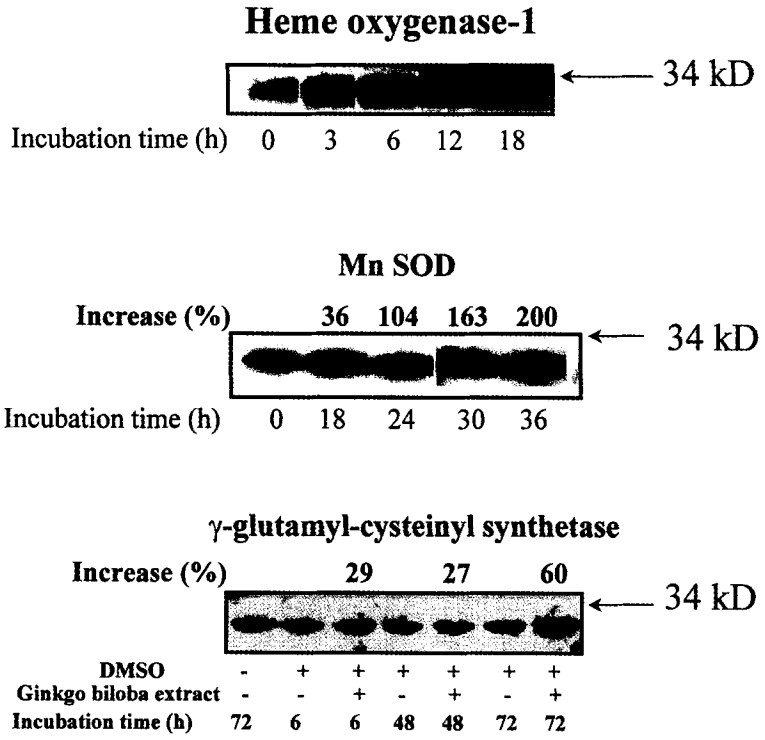

FIGURE 5 Induction of antioxidant proteins, HO-1, manganese superoxide dismutase (MnSOD) and the regulatory subunit of γ-glutamyl cysteinyl synthetase (GCSr) in T-24 cells by the extract of Ginkgo biloba leaves. The proteins were detected on membranes by incubation with the protein specific antisera as described in the methods. Time dependent increase (% of control) in the abundance of MnSOD and GCSr were semi-quantitated by densitometric analysis of images on autoradigraphic films

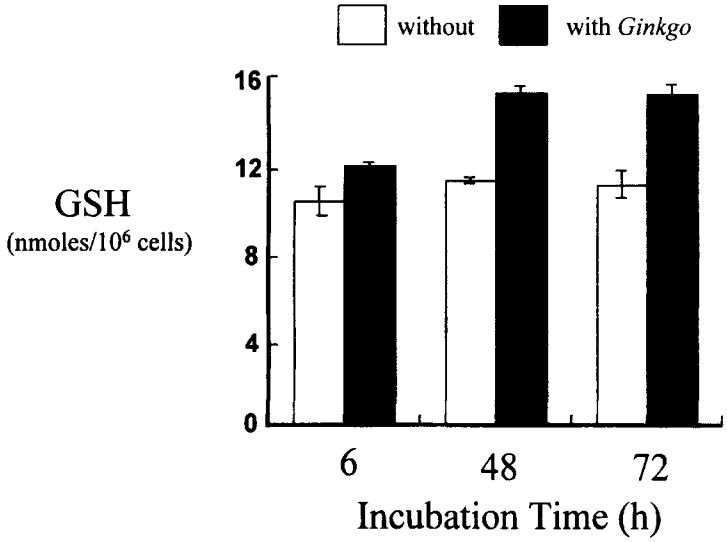

FIGURE 6 Cellular glutathione is elevated by the incubation of T-24 cells with the extract of Ginkgo biloba leaves. T-24 cells were incubated with either the vehicle (0.1% DMSO) or with the extract of Ginkgo biloba leaves for 6, 48, and 72 hours as described in the Methods

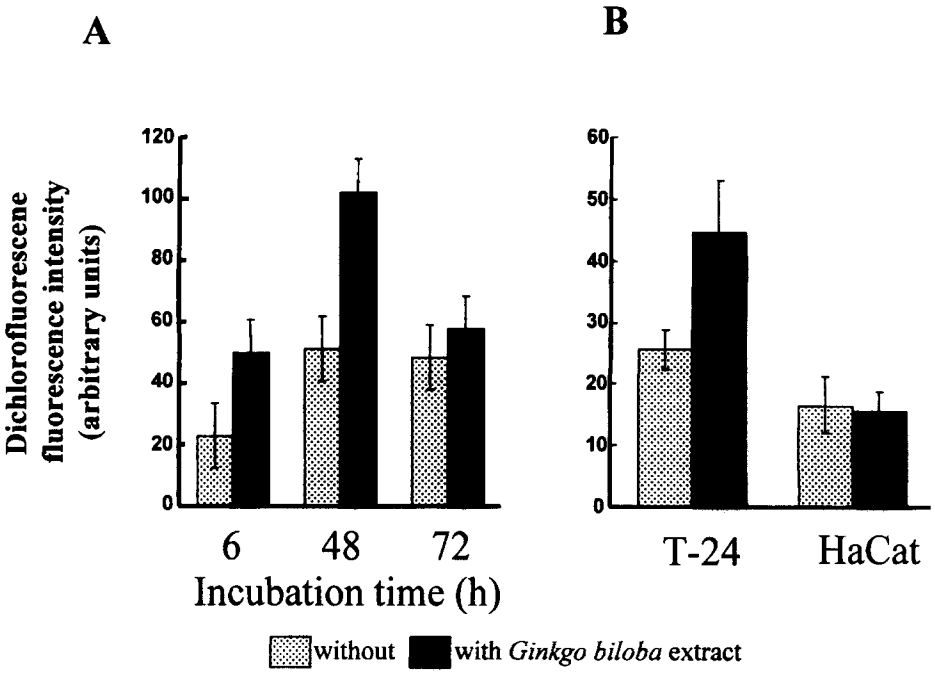

FIGURE 7 Augmentation of intracellular peroxides by Ginkgo biloba extract in human bladder cancer cells. A T-24 cells were incubated with the extract for the 6, 48 and 72 hours. The intracellular accumulation of peroxides was assayed by the oxidation of DCF as described in the methods. Increase in peroxides was seen as early as 6 hours and peaked at 48 hours in the presence of the extract. B Intracellular peroxides in human bladder cancer cells were higher than those in human keratinocyte cell line (HaCaT). Peroxide levels in T-24 cells were increased after 24 hour incubation with the extract. This was not seen HaCaT cells suggesting a selective effect of *Ginkgo biloba* extract on human cells in culture

TABLE IV *Ginkgo biloba* extract sensitive genes encoding proteins for repair and synthesis of DNA and proteins for cell cycle. The transcripts were identified by "Difference Call" analysis as mRNAs whose expression was affected by two-fold or more in the presence of the extract compared to untreated control cells. The abundance of each transcript in untreated control T-24 cells and in Ginkgo biloba extract treated cells, at the three incubation periods, is shown by fluorescence intensity

			Fluoresence intensity		
Name of Gene	*Accession #*	*control*	*6 hours*	*48 hours*	*72 hours*
DNA ligase 1	M36067	130	168	221	229
DNA fragmentation factor	U91985	189	425	210	259
SMT 3B	X99585	505	1057	1035	1097
topoisomerase-I	U07804	103	182	189	165
subtelomeri	U06155	20960	14255	10467	10479
proliferating cell nuclear antigen	J05614	1581	2454	3227	2804
helicase like protein	U09877	558	345	382	247
radiation inducible early immediate gene	S81914	2463	2492	3505	4585
DNA polymerase delta subunit	U21090	898	776	1217	1825
DNA mismatch repair	U07418	609	823	995	1507
meiotic recombination	U37359	221	154	259	506
EB1	U24166	1467	1549	2358	4056
cyclin B	M25753	751	805	1366	2001
cyclin D	M92287	123	207	293	716
cdc2	X05360	105	171	229	259
CDK4	U37022	949	860	1173	2013

Ginkgo biloba extract did not affect cell proliferation in a panel of 60 different human cancer cell lines screened by The National Cancer Institute showing that the lack of effect of the extract on proliferation of T-24 cells is present in a large number of different cancer cells. In the absence of any significant cell loss shown by the flow cytometry and cell proliferation data, inhibition of thymidine incorporation suggests inhibition of DNA damage by the extract and the augmentation of DNA repair mechanisms, as suggested by increase in transcripts encoding proteins essential for repair of damaged DNA (Table IV).

DISCUSSION

The primary aim of this study was to evaluate the potential molecular targets of a botanical extract, with broad claims of therapeutic benefits, by the application of recently developed techniques for exploring global gene expression profiles. Analytical strategies that utilize well defined *in vitro* assays of cellular responses can generate useful information for designing the more challenging *in vivo* studies. This is best illustrated by the Developmental Therapeutics Program of National Cancer Institute [12,13], which has completed *in vitro* screening of more than 70 000 compounds, including botanical extracts. This strategy enabled the identification of ~ 1000 candidate compounds which inhibit proliferation of cancer cells in which p53 is mutated [12]. A similar analysis of the expression pattern of 1,400 genes in response to different anticancer drugs enabled classification of the same 60 cancer cell lines into responders and non-responders [13]. Such data may prove useful

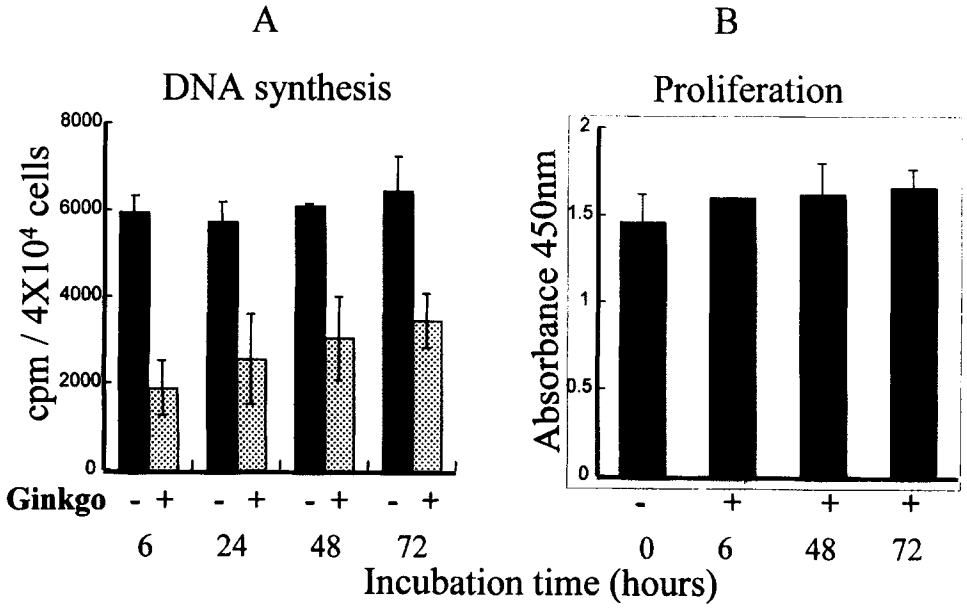

FIGURE 8 Inhibition of DNA synthesis without affecting proliferation of human bladder cancer cells by the leaf extract of Ginkgo biloba. Incorporation of ^{3}H-thymidine into trichloroacetic acid insoluble fraction of the cells was inhibited as early as 6 hours and remained low through the course of the experiment in the presence of the extract. Metabolic assay (reduction of tetrazolium dye, see Methods) for cells treated identically, except without ^{3}H-thymidine, showed no effect on proliferation of the cells by the extract

in making a rational choice of drugs to target specific cancers at their specific stage of malignancy. The extract of *Ginkgo biloba* leaves is an attractive candidate for detailed investigation because of the potential pharmacological activities of flavonoid-glycosides and terpenoids present in the extract and its popular use antioxidant dietary supplement. Human bladder cancer cells with activated, oncogenic G-protein, Ha-ras were selected as target cells because of the pro-oxidant effects of some of the ras family members [15,46] which may play a causative role in the disease processes.

The application of high density oligonucleotide chips to define the changes in the mRNA profile of a cancer cell under oxidative stress has revealed a novel action of *Ginkgo biloba* extract. The transcripts for heme-oxygenase-1 (HO-1) and mitochondrial superoxide dismutase (MnSOD) were induced as were their respective proteins. HO-1 has previously been shown to be regulated by controlling its transcription under several conditions including elevated levels of intracellular peroxides [47]. Heme oxygenase-1 catalyzes the oxidation of heme to carbon monoxide, iron, and biliverdin, a potent antioxidant [41]. HO-1 has also been reported to result in growth arrest and an increased resistance to hyperoxia [48]. Another antioxidant enzyme, MnSOD, is expressed at decreased levels in cancer cells [49] and it has been suggested that overexpression of human MnSOD suppresses growth of cancer cells [50]. Since *Ginkgo biloba* extract induced HO-1, Mn SOD and inhibited DNA synthesis, the extract may play an important role in determining the fate of the extract sensitive cells.

Selective expression of genes related to antioxidant functions showed a "signature" of an adaptive response to oxidative stress. Intracellu-

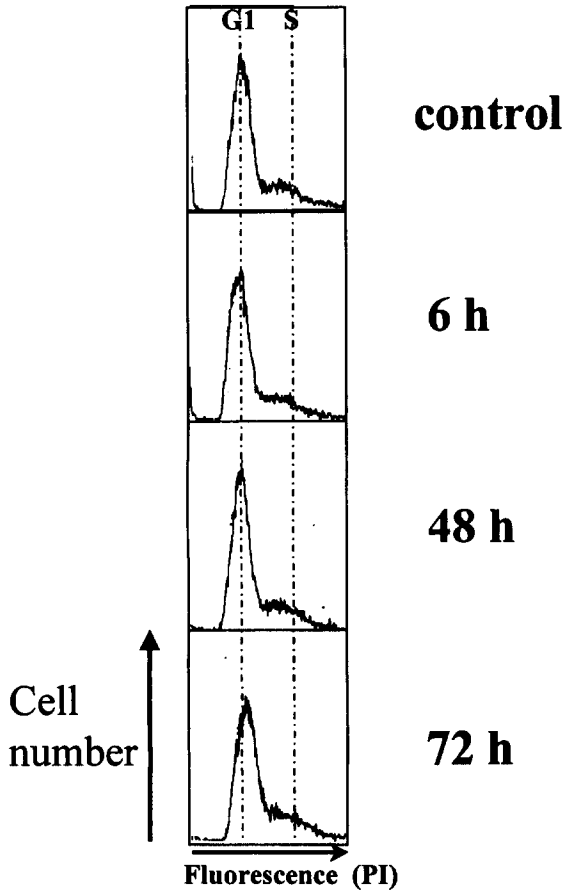

FIGURE 9 Flow cytometric analysis of fixed, permeablized and propidium iodide stained T-24 cells. Incubation of human bladder cancer cells with the extract from Ginkgo biloba leaves did not affect the fractions of cells in G1 and DNA-synthesis (S) phases of the cell cycle

lar peroxides accumulated in the cells treated with the extract as shown above (Figure 6). This was a surprising observation because the extract has been shown to be antioxidant in a variety of *in vitro* assays where its action is attributed to scavenging of reactive radicals or chelating pro-oxidant transition metal ions [23]. However in cell-free systems, in the presence of transition metals such as Cu^{++}, flavonoids, one of the major known components of the extract, can also act as oxygen radical generators [51]. In T-24 cells this pro-oxidant mechanism is less likely to operate because two transcripts encoding proteins for the chelating of iron and other transition metals were present in high abundance; these were the transcripts for ferritin light chain and metallothionein. The transcript for the latter was the highest expressed mRNA in T-24 cells under basal conditions. High basal expression of metallothionien gene supports the proposal that T-24 cells with activated Ha-ras have high basal levels of reactive radicals since the latter have previously been shown to be potent activators of metallothionein gene [18].

In T-24 cells the increase in intracellular peroxides is more likely to be attributed to activation of membrane NADPH oxidase by Ha-ras [15] and possibly, ras related GTP binding proteins. As noted above the transcripts encoding each of these ras proteins were present in large abundance. Ras proteins encoded by each of these transcripts have a highly conserved carboxyl terminal domain, which is a putative substrate for farnesyl transferase [52], the transcript for which was increased by 2.6 fold after 6 hour incubation with the extract (see Supplemental Data). Farnesylation of ras is essential to interact with and activate membrane NADPH oxidase; the T-24 cells also showed high abundance (~30% of GAPDH) of the transcript for neutrophil cytochrome b light chain, an essential subunit of NADPH oxidase.

Reactive oxygen species and mitochondrial dysfunction have been implicated in the initiation and propagation of apoptosis [53,54]. Transfection of normal tumor suppressor, p53, in a colorectal cancer cell line with defective p53 alters the expression of 7,202 transcripts [53]. Some of these transcripts that are markedly increased (ten-fold or greater), encode proteins that either generate reactive oxygen species or act as antioxidants. The induction of these transcripts is followed by depletion of glutathione, increase in reactive oxygen species and initiation of apoptosis. Although T-24 cells generated peroxides in the presence of the extract, intracellular glutathione was not oxidized and on the con-

trary, there was a ~35% increase in intracellular glutathione. None of the "antioxidant-transcripts" induced by *Ginkgo biloba* extract identified with p53 induced transcripts suggesting potential of a human cell to elicit stimulus specific antioxidant response.

Global transcriptional analysis of cells such as in *Saccharomyces cerevisiae* has shown that a change in the status of a single nutrient such as glucose can alter the expression of ~ 2000 genes, ~ 30% of the yeast genome [10]. When *Saccharomyces cerevisiae* were exposed to a DNA damaging agent the expression of ~ 400 genes out of ~6200 (all of the expressed yeast genome) was altered showing that a large fraction of the yeast genome is responsive to an agent that threatens its integrity [55]. In an example more comparable to the present study, human fibroblasts were exposed to fetal calf serum, a complex mixture of bioactive compounds from heterologous species [56]. Global transcriptional analysis of 8600 distinct human fibroblast transcripts detected changes in the expression of ~500 genes. The study identified previously recognized transcription factors and revealed the coordinated expression of numerous genes which were not known to be regulated by serum. The description of serum responsive transcriptional program of human fibroblasts complements our findings in a human cancer cell line exposed to a complex phytochemical extract.

The discussion of the extract sensitive transcriptional response is focused on groups of functional categories with five or more related genes, such as antioxidant genes or genes encoding vesicular transport proteins. However changes in the expression of a number genes which did not form such a group and listed in the Supplemental Data have potentially important functions. One such example is the induction NADP dependent leukotriene dehydrogenase by the extract of *Ginkgo biloba* leaves. The enzyme plays an important role in anti-inflammatory response by inactivating leukotriene B_4 [57], a potent chemotactic and proinflammatory factor produced by many tissues. Additional evidence that the cancer cells are eliciting a selective transcriptional response to the presence of the extract is the expression of genes associated with an adaptation to heat-shock. Induction of heme oxygenase-1 has been noted above. Another noteworthy transcript that was induced at 6h was that for p23, which has been shown to hetero-dimerize with heat shock proteins hsp70 and hsp90 [58], both of which play an important role in regulating protein folding. Lamin B, a nuclear protein has recently been identified as a "prompt" heat shock protein [59]. In the presence of the extract of *Ginkgo biloba* there was a 2-fold increase in the transcript for lamin B in the cancer cells.

The goal of this study was to determine a profile of genes that are targeted by *Ginkgo biloba* extract and the application of the Affymetrix Hu6800 high density oligonucleotide GeneChips have now identified one such profile. Analysis of the gene expression profile of the human cancer cells in response to the extract uncovered a coordinated transcriptional strategy that is utilized to enhance the cell's ability to tolerate oxidative stress. Many of the genes listed in the Antioxidant/heat shock/mitochondrial category (Table II) have antioxidant response elements (ARE) in their promoter regions; AREs are also known as electrophile responsive elements and nuclear respiratory factor element-2 (Nrf2), For example, HO-1 gene contains five AREs [60], and AREs are present in the 5′ flanking regions of the regulatory subunit of GCS [61], and mitochondrial genes [62,63]. These promoter regions also have consensus sequences for 12-O-tetradecanoyl phorbol-13-acetate responsive element, a binding site for activation protein-1 [18]. The GeneChip data from the control and extract treated cells were re-examined for the possible changes in the abundance of transcripts for Nrf2 and Nrf1. The data showed that the abundance of Nrf2, but not that for Nrf-1, increased by 65% over the 72h incubation. A novel feature of this adaptation is the potential recruitment of the

Golgi system and intracellular transport of vesicular proteins during the adaptive response. Perhaps the transcription of the genes for the assembly and transport of intracellular vesicles is also regulated by promoters similar to those of genes encoding antioxidant proteins

The transcriptional response described here partially defines the potential of the flavonoid and terpenoid containing extract of *Ginkgo biloba* leaves for regulating the expression of genes encoding antioxidants, mitochondria, the Golgi system and for the repair and synthesis of DNA in a human cancer cell. The application of reliable techniques that measure global, cellular and molecular responses of selected cells *in vitro* can begin to delineate the specific biological effects of natural extracts and offer a more rational approach to design *in vivo* studies.

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