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Association of Cellular Thiol Redox Status With Mitogen-Induced Calcium Mobilization and Cell Cycle Progression in Human Fibroblasts

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Abstract Human gingival fibroblast cultures were used to investigate the role of cellular thiol redox status in the mitogenic response. Increases in intracellular Ca^{2+} and cell cycle progression beyond G1 were followed as parameters of cellular mitogen-induced responses. Ethionine provided a G1 stage synchronization and altered the cellular redox poise as measured by the ratio NAD(P)H/NAD(P)$^+$. Cultures harvested immediately after the 6 day ethionine low-serum synchronization showed a significant oxidation of their redox poise. Synchronized cultures, which were also glutathione (GSH) depleted, still showed an oxidized redox poise and significantly reduced GSH levels following a 24 hr incubation in drug-free, rich medium. Cellular reduced nicotinamide nucleotide levels correlated strongly ($r = 0.995$) with capacity to mobilize intracellular Ca^{2+} in response to basic fibroblast growth factor (bFGF). The sustained mitogenic response, as determined by cell cycle progression beyond G1, was also found to be interrelated with the cellular thiol redox status. Following a 24 hr recovery incubation in serum-rich medium, formerly synchronized cultures showed a rebound of their redox poise to a more reduced state and significant cell cycle progression beyond G1. In contrast, synchronized, GSH-depleted cultures did not progress and showed population distributions similar to those of cultures harvested immediately postsynchronization. Upon recovery of cellular GSH and reduced nicotinamide nucleotide levels, formerly GSH-depleted, growth-arrested cultures resumed cell cycle progression. The results suggest that the cellular response to specific mitogens is interrelated with the cellular thiol redox status. Cells that possess a thiol redox status below a threshold response point may have compromised Ca^{2+} sequestration and/or mobilization and therefore may be incapable of initiating the mitogen induced response cascade that culminates in cell cycle progression.

Key words: cell cycle kinetics, nicotinamide nucleotides, glutathione, mitogen responsiveness, cation mobilization

The loss of regulatory controls and aberrant cellular growth patterns represent one of the hallmark features of the neoplastic process [1–4]. Clarification of the mechanisms involved in normal cell cycle regulation may provide insight into the growth deregulation that occurs in neoplasia. Normal cell cycle regulation is a complex process [5,6]. Cells progressing in the cell cycle coordinate and transmit signals from extracellular sources, e.g., growth factors and mitogens, into intracellular responses, including secondary messengers and regulation of gene expression [7,8].

One of the putative intracellular secondary messengers of the mitogen-induced response is calcium (Ca^{2+}) [9,10]. Mitogen-receptor binding to the cell membrane releases both diacylglycerol and inositol 1,4,5-triphosphate, which appear to mobilize intracellular Ca^{2+} stores [11–15].

Intracellular free Ca^{2+} concentrations transiently increase to appreciably higher levels than resting intracellular free Ca^{2+} following mitogen binding [16–18]. It has been proposed that reduced nicotinamide nucleotides, or a group in close redox communication, as membrane-bound thiols, function in the regulation of divalent cation channels [19–22]. Reduction of the mitochondrial nicotinamide nucleotide pool by B-hydroxybutyrate was shown to promote Ca^{2+} retention, whereas oxidation by oxaloacetate caused efflux [19,20]. The cellular thiol status is interrelated with the cell redox state via the glutaredoxin, thioredoxin, and glutathione (GSH) reductase pathways [23–25].

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In vivo, cells are continually exposed to mitogen-rich serum but only respond in a proliferative fashion during specific times of the cell cycle [2,5,26]. Apparently, cell cycle progression is regulated, at least in part, by intracellular parameters present in G1 cells that determine cellular mitogenic responsiveness [2,5,26].

The purpose of this study was to evaluate whether cellular thiol status/redox poise functions as a determinant in how cells respond to mitogens. The cellular concentration of the primary intracellular free thiol GSH was determined as a measure of cellular thiol status. GSH is a tripeptide that functions in cytoprotection, maintenance of protein thiol status, and modulation of enzyme activity via disulfide interchange [27–29]. We have recently reported that depletion of cellular GSH slows recovery from a G1, growth-arrested state [30].

Two distinct parameters of the cellular mitogenic response were followed. To evaluate cellular capacities for mitogen-receptor interaction (immediate response), fibroblasts were challenged with human basic fibroblast growth factor (bFGF) and changes in intracellular free Ca^{2+} monitored. Cellular sustained responsiveness toward mitogenic stimuli was determined by following cell cycle progression of G1 synchronized cultures from G1 to S via flow cytometric DNA analyses.

MATERIALS AND METHODS

Cell Culture

As part of an ongoing study to assess the protective role of GSH in the oral cavity, human gingival normal fibroblast explants were obtained via biopsy, from six periodontally and systematically healthy volunteers, who had remained medication free for at least 1 week prior to biopsy. Following palatal block anesthesia (to alleviate any tissue disruption from local anesthetic infiltration), the gingiva was harvested from a specified site, i.e., interdental papilla between the maxillary first molar and second premolar.

Gingival fibroblast cultures were grown in a modification of Eagle minimum essential medium (EMEM) (Gibco, Grand Island, NY), formula 78-5048. This growth medium ("B") consisted of EMEM with the addition of 1.5× essential amino acids, 1.5× vitamins, 2× nonessential amino acids, and approximately 1× l-glutamine. Fetal bovine serum (FBS) (Gibco) was added to the medium for a final concentration of 10% ("B-10"), except during the "synchronization" protocol, in which the serum concentration was decreased to 0.3%. Following the establishment of primary cultures, the fibroblasts were grown in antibiotic-free B medium.

At passage, and at cellular harvesting, the cultures were first rinsed with phosphate-buffered saline (PBS). Then detached from the substrate with equal volumes of 0.02% EDTA-PBS and 0.01% trypsin PBS (prepared from lyophilized trypsin; Worthington Biochemicals, Freehold, NJ). All experiments were conducted on cultures that had undergone no more than 20 population doubling levels (PDL).

Ethionine-Mediated Cell Synchronization and GSH-Depletion Protocols

Fibroblast cultures were synchronized by a modification of the protocol of Wilke et al. [31,32]. Specific cultures were GSH depleted by a protocol combining treatment with 2 cyclohexene-l-one and buthionine sulfoximine as reported in our recent publication [30]. Fibroblast culture experimental groups evaluated were: 1) log growth controls (L); 2) synchronized controls, harvested immediately *after the 6 day* ethionine synchronization (S); 3) synchronized controls, harvested after an additional 24 hr in B-10 (S 24); 4) experimental, synchronized, GSH-depleted, harvested after an additional 24 hr in B-10 (S-D 24); and 5) experimental, synchronized, GSH-depleted, harvested after an additional 40 hr in B-10 (S-D 40).

GSH Determination

Total GSH and GSSG levels were determined in accordance with the method of Eyer and Podhradsky [33].

Protein Concentrations

Proteins were determined by the Lowry et al. [34] method.

Determination of Total and Reduced Nicotinamide Nucleotides

For reduced and total nicotinamide nucleotides, the samples were resuspended to 0.5 ml with ice-cold 0.02% EDTA-PBS (pH > 10.5, adjusted with KOH). In contrast, the oxidized sample, which served as an internal standard control, was resuspended to 500 μl in a 0.4 M MES buffer, pH 6.02, that contained 6×10^{-4} M oxaloacetate. To induce cellular decompartmen-

talization, the samples were transferred to cryovials, immersed in liquid N_2, then rapidly thawed and vortexed vigorously.

Next the reduced and total samples were resuspended to a final volume of 1 ml in the "redox buffer" and the "modified redox buffer," respectively. The redox buffer contained 10 mM nicotinamide, 20 mM $NaHCO_3$, and 5% Triton X-100 (Calbiochem, La Jolla, CA), pH 10.62. The modified redox buffer also contained 20 mM B OH butyrate, 5 μM rotenone, 20 mM ETOH, and 0.2 mM aminooxyacetate. The redox samples were either assayed immediately or wrapped in foil and stored overnight in liquid N_2.

Redox Sample Preparation

Prior to being assayed, the redox samples were heated for 20 min in a 60°C water bath to degrade the oxidized nicotinamide nucleotides. Protein was extracted with 2 M $HClO_4$, and the samples were centrifuged to remove the acid precipitated protein. After centrifugation, the pH of the samples was immediately adjusted to *approximately* 10.60 with 2 M KOH.

The nicotinamide nucleotide redox state assay was conducted on a SLM-Aminco DW 2 C dual-wavelength spectrophotometer using 340 − 370 nm. NAD(P)H levels were determined by comparison to a 12 point standard curve. The NADPH (N 7505; Sigma, St. Louis, MO), standards were prepared fresh in the redox buffer and ranged from 2.5 to 100 nmol/ml. The assay was conducted in the linear portion of the curve ($r \geq 0.999$). The oxidized sample functioned as an internal standard control, to account for any NAD(P)H spectral changes that were attributable to the presence of cytoplasmic components.

Ca^{2+} EGTA Buffers

Calcium-EGTA buffers were prepared according to the specifications of the computer program designed by Fabiato [35,36]. The calcium-EGTA buffer mixes contained 20 mM HEPES (K^+, pH 7.2), 120 mM KCl, 1 mM $MgSO_4$, 1 mM EGTA, and specified amounts of CaCl to make eight solutions ranging from 56 to 1,000 nM free Ca^{2+}. A calcium-sensitive electrode was used to verify the free Ca^{2+} concentrations [37].

In Situ Calibration and Collapse of Intracellular Gradients

Since the properties of fluorescent probes are affected by the intracellular environment and the presence of partially hydrolyzed forms, the dissociation constant (Kd) was determined on the fura-2 trapped in intact cells [38]. After harvesting and centrifugation, fibroblast pellets were resuspended in 0.1% bovine serum albumin and loaded with dye by an incubation with 1 μM fura-2 AM (Molecular Probes, Eugene, OR) for 45 min at 37°C, 5% CO_2. The fura-loaded cells were then washed twice in Ca^{2+}- and chelator-free PBS.

The changes in fluorescence at 345 and 380 nm (510 nm emission) were recorded for fura-2-loaded cells (3×10^6 cells/ml) suspended in the calcium-EGTA buffer mixes in the presence of ionophores and metabolic inhibitors (20 mM NaN_3, 10 mM 2-deoxyglucose, 0.5 μg/ml nigericin, 2 μM ionomycin, and 2 μM carbonyl cyanide M-chloroprophenyl hydrazone). These reagents collapse the intracellular gradients and equilibrate Ca^{2+} and have been shown not to affect the fluorescence of a related fluorochrome, indo-1 [18].

Ca^{2+} was allowed to equilibrate during a 3 hr incubation in an Orbit Microprocessor Shaker Path (Lab Line Instruments, Melrose Park, IL) set at 37°C and 100 rpm. After equilibration, the pH of the in situ standards was determined, and, if necessary, readjusted back to pH 7.2.

A Kd of 128 nM was determined using the equation

$$\text{Kd} = \frac{[Ca^{2+}]}{[(R - \text{Rmin})/(\text{Rmax} - R)]} \times (B),$$

where R = 345/380 ratios for cells with collapsed gradients suspended in the calcium-EGTA buffer mixes, Rmin and Rmax = the 345/380 ratios obtained in the presence of excess EGTA and calcium respectively, and

$$B = \frac{\text{excitation 380 min}}{\text{excitation 380 max}} \text{ [37].}$$

The values for Rmin and Rmax were determined in fura-loaded, Triton-lysed cells suspended in no Ca^{2+} (min) and free $[Ca^{2+}] > 10$ μM (max). In the absence of cells, the Kd for fura-2 was determined by the above procedure to be 167 nM (data not shown).

Fluorimetry

Readings of the in situ cellular standards were conducted on a Perkin Elmer LS-5B luminescence spectrophotometer with the excitation and emission band width at 10 mm. The standards

and cuvette holders were maintained at 37°C by a circulating water bath, and the samples were constantly stirred by a tapered-top cuvette stirring system (Instech Labs, Plymouth Meeting, PA).

The in situ calibration curve was conducted several times to ensure reproducibility. The free $[Ca^{2+}]_i$ of resting fibroblasts was determined to be 98, 93, and 101 nM on three separate evaluations; these values closely approximated reported resting $[Ca]_i$ levels of 100 nM [16]. Ca^{2+} mobilization experiments were then run at identical instrument settings. Cellular samples were evaluated to determine the daily Rmin and Rmax in conjunction with every Ca^{2+} mobilization assay. Free Ca^{2+} was determined using the ratio method of Grynkiewicz et al. [37].

Ca^{2+} Mobilization Assay

Cell samples were obtained from all the culture growth regimines, i.e., L, S, S 24, S-D 24 to assess cellular responsiveness to the mitogen, bFGF, by monitoring $[Ca^{2+}]_i$. Cell loading with fura was conducted as in the in situ protocol. Two separate parameters of the Ca^{2+} mobilization response were evaluated. To determine cellular capacity to respond with intrinsic Ca^{2+} stores, fura-loaded cells were resuspended in Ca^{2+}-free PBS. Ratio readings were first taken to determine sample initial levels of $[Ca^{2+}]_i$. Then, the samples were challenged with bFGF at a dose of 50 ng/ml. Ratio readings were recorded immediately after addition of bFGF and every 30 sec thereafter for 7 min.

Because part of the Ca^{2+} mitogenic response is thought to involve cellular ability to mobilize Ca^{2+} from both intracellular and extracellular sites, fura-loaded cells were also suspended in a "Ca^{2+} mobilization buffer" [16]. This buffer contained 10 mM K-HEPES (pH 7.2), 145 mM $NaCl_2$, 5 mM KCl, 1 mM $MgSO_4$, 0.5 mM Na_2HPO_4, 5 mM glucose, and physiologic extracellular concentrations (1 mM) of $CaCl_2$. Cell samples were incubated at 37°C for 45 min in this buffer, unstimulated Ca^{2+} levels recorded, and then these cells were challenged with bFGF and the free Ca^{2+} ratios recorded.

Human bFGF was selected as the mitogenic stimulus for several reasons. bFGF has been shown to induce Ca^{2+} mobilization from numerous cultured cells, including fibroblasts [39,40]. Furthermore, in that these cultures are human cells, the species compatibility should be reflected in appropriate receptor expression.

Cell Preparation for Flow Cytometry and DNA Staining Procedures

Cell nuclear preparations and DNA staining were conducted in accordance with the methods of Larsen et al. [41]. Cells were harvested, suspended in PBS, and centrifuged at 500*g*, 4°C, for 10 min. The pellet was resuspended to 2 ml in a phosphate (6.5 mM Na_2HPO_4, 2.7 mM K H_2PO_4, 137 mM NaCl, 0.5 nM EDTA, pH 7.2)—0.1% Triton buffer and placed in an ice bath for 5 min. The cells were then lysed via *tube inversion.* Fixation of the nuclear preparation was done by addition of 0.7 ml of a 4% formaldehyde solution. Samples were stored at 4°C for <12 hr prior to flow cytometry.

To remove RNA, the nuclei were incubated on the day of staining for 20 min in a 37°C H_2O bath with a final concentration of 150 U/ml of RNAase (RNAase A; Worthington Biochemicals). Prior to centrifugation, the nuclei were vortexed (to remove cytoplasmic tags) and passed through a 53 μm nylon mesh filter (Spectra/Mesh; Spectrum Medical Industries, Inc., Los Angeles, CA) to remove cellular debris.

Finally, the nuclei were suspended in 50 μg/ml propidium iodide (PI) (Sigma) and incubated overnight at 4°C. Flow cytometry was conducted the following morning.

Flow Cytometry

The DNA content of the PI-stained nuclei was analyzed on an Ortho System 50H Cytofluorograf (Bectin Dickinson & Co.) at a flow rate of <300 particles per second. The nuclei were suspended in a phosphate-0.1% Triton buffer at a concentration of approximately 10^6/ml. The carrier sheath fluid was phosphate-buffered normal saline. Excitation output was 200 mW from the 488 nm line of a Spectra Physics 164-05 argon ion laser in light mode. The PI emission was collected through a 630 long-pass filter and analyzed on an Ortho 2150 Data Acquisition System (Data General Nova 4 CPU, running MPOS). Prior to each run of samples, instrument linearity and calibration were verified with fluorescent microspheres (PolySciences, Inc.). Repeatability over time was confirmed with PI microbead standards (Flow Cytometry Standards Corp., Research Park, N.C.) as an experimental internal standard. PI-stained fixed calf thymocyte nuclei confirmed a 2:1 (G2/M:G1-G0) DNA index. The distribution of nuclei in specific cell cycle phases (G0/G1, S, G2/M) was derived

from the area of the red fluorescence histograms, assuming a gaussian distribution of the G-G1 and G2/M about their maxima and attributing the inclusive residual DNA histogram segment to S phase nuclei. Debris, aggregates, and doublet nuclei (which falsely increase the G2/M population) were excluded from the DNA histogram by first plotting fluorescence pulse peak vs. pulse area as a bit map, two-parameter cytogram and using a logic gate to exclude nonconforming pulses.

RESULTS

Results from our earlier study established that our GSH depletion protocol was both specific and reversible [30]. Culture levels of GSSG remained nondetectable both immediately post GSH depletion and at the 24 hr cell harvest, implying that formation of protein mixed disulfides did not occur as a result of the GSH depletion protocol [30].

In that study, we also demonstrated by ^{3}H-thymidine incorporation and flow cytometry that the fibroblast cultures were in a G1 growth arrested state with $\geq 80\%$ of the treated cells in G1 following the 6 day ethionine synchronization protocol [30]. Furthermore, the reversibility of the synchronization and GSH depletion protocols was confirmed by both ^{3}H-thymidine incorporation and flow cytometric DNA analyses [30]. The ethionine synchronization and GSH depletion protocol effects on DNA distribution can be seen from the representative histograms presented in Figure 1.

Preliminary studies were conducted to determine whether our fibroblast strains possessed the esterase necessary to hydrolyze the fura-2 AM. Resting, fura-loaded cells showed an excitation peak at 345 nm. Following cell lysis with 0.1% Triton X-100, the excitation spectra in the presence and absence of 5 mM EGTA were consistent with the fura-2 anion, indicative of deesterification of the AM. In the absence of EGTA, there was an increase in excitation at 345 nm; with EGTA, the excitation peak shifted to 380 nm. Furthermore, the $[Ca^{2+}]_i$s of resting fibroblasts were evaluated and were determined to be 98, 93 and 101 nM on three separate trials (data not shown). As a final preliminary control study, 15 μl aliquots of a 1 mg BSA/ml PBS solution (the bFGF solvent) were added to fura-loaded cells during constant stirring, and the $[Ca^{2+}]_i$ ratios were monitored. No alteration in $[Ca^{2+}]_i$ (determined by 345/380 vs. 510 nm ratios) occurred after the addition of the BSA in PBS solution (data not shown).

As would be anticipated based on ethionine's proposed mechanism of action, nicotinamide nucleotide assay results demonstrated that the ethionine synchronization protocol perturbed the cellular redox state (Table I). The altered redox poise (relative to log growth control cultures) observed in our synchronized cultures was both transient and reversible. Ethionine-synchronized cultures incubated for an additional 24 hr in B-10 (group S 24) showed a rebound of culture redox poise to a more reduced state (43.9% ± 13.4% oxidized to 15.7% ± 3.7% oxidized) and a decrease in total nicotinamide nucleotide levels (12.8 ± 0.70 nmol/mg protein to 10.5 ± 1.2 nmol/mg protein) to distributions approximating those seen in log growth cultures (group L) (10.0 ± 1.3 nmol/mg protein). In contrast, the redox state and total nicotinamide nucleotide levels of the synchronized, GSH-depleted cultures (group S-D 24) did not deviate from the levels obtained from the synchronized control cultures (group S), despite an additional 24 hr incubation of the S-D cultures in B-10.

Total nicotinamide nucleotide levels were elevated in both the synchronized control (group S) (12.8 ± 0.7 nmol/mg protein) and the synchronized, GSH-depleted (Group S-D 24) (12.1 ± 1.0 nmol/mg protein) cultures relative to levels obtained from log growth cultures (group L) (10.0 ± 1.3 nmol/mg protein). Comparison of total nicotinamide nucleotide levels, group L vs. group S, is significant by Student's t test ($P < 0.05$).

Relative to log growth (L) cultures, (9.9% ± 5.1% oxidized), oxidation of the cellular redox state was noted in both the S and S-D cultures (43.9% ± 13.4% and 42.5% ± 9.5%, respectively). Furthermore, these differences were statistically significant; the percent redox poise oxidation L vs. S was significant at the $P < 0.01$ level, L vs. S-D 24, $P < 0.05$, Student's t test.

The two time course GSH depletion experiments provided information regarding culture redox poise response to the GSH depletion protocol. S-D O (cultures harvested immediately after GSH depletion) showed a percent oxidation lower than that seen in S-D 24 cultures, which were harvested at 24 hr (29.8% oxidation vs. 42.5%). Therefore, the cellular redox poise oxidation increased during the 24 hr following the GSH depletion. S-D 40 cultures (harvested 40 hr after GSH depletion) showed both a decrease in per-

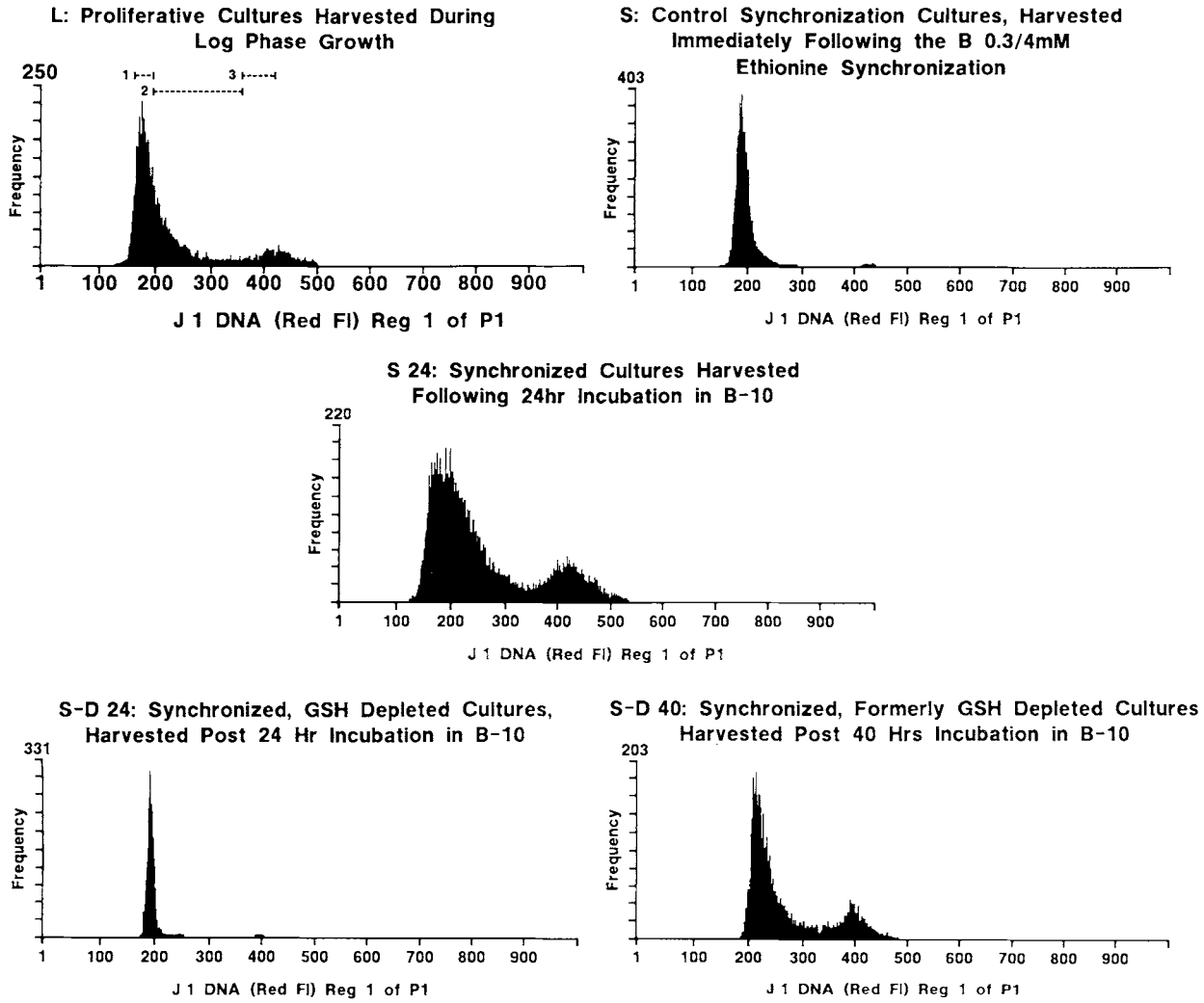

Fig. 1. Representative histograms of DNA-related fluorescence of propidium iodide-stained human gingival fibroblast nuclei that were harvested following: L, log phase growth; S, 6 day ethionine synchronization protocol; S 24, 24 hr incubation in rich medium after synchronization; S-D, 24 hr incubation in rich medium after synchronization and glutathione (GSH) depletion; S-D 40, 40 hr incubation in rich medium after synchronization and GSH depletion.

TABLE I. Distribution of Nicotinamide Nucleotides (nmol nicotinamide nucleotides/mg protein ± S.D.)

Group (n)[a]	NAD (P) H + NAD (P)$^+$ total	NAD (P) H reduced	NAD (P)$^+$ oxidized	Percent oxidized
L (4)	10.0 ± 1.3*	9.0 ± 1.6	1.0 ± 0.4	9.9 ± 5.1*,**
S (3)	12.8 ± 0.7*	7.2 ± 2.1	5.6 ± 1.6	43.9 ± 13.4**
S 24 (3)	10.5 ± 1.2	8.8 ± 0.7	1.5 ± 0.3	15.7 ± 3.7
S-D 24 (3)	12.1 ± 1.0	6.9 ± 1.3	5.1 ± 1.3	42.5 ± 9.5*
S-D 0 (1)	13.7	9.6	4.1	29.8
S-D 40 (1)	9.0	6.4	2.6	29.1

[a]L, log growth controls; S, synchronized controls, harvested immediately after the 6 day low-serum/ethionine synchronization; S 24, synchronized controls, harvested after an additional 24 hr in B-10; S-D 24, synchronized, GSH-depleted, harvested after 24 hr in B-10; S-D 0, experimental, synchronized, harvested immediately after the GSH depletion; S-D 40, experimental, synchronized, harvested 40 hr after GSH depletion.

*$P < 0.05$.

**$P < 0.01$.

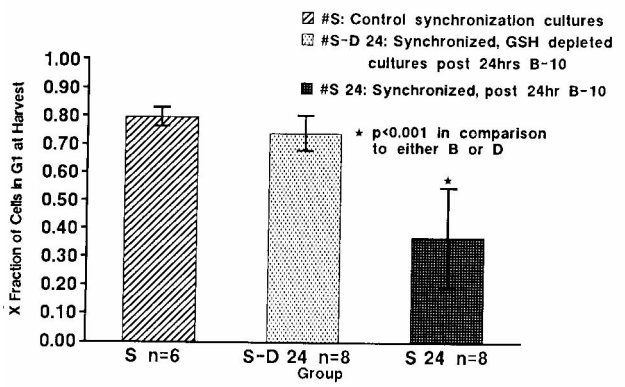

Fig. 2. Bar histogram depicting cell cycle distribution (as $\bar{X}$ fraction ± SD of cell population in G1) of human gingival fibroblasts that were harvested for flow cytometric analysis after: group S, ethionine synchronization protocol; group S-D 24, 24 hr incubation in rich medium after synchronization and GSH depletion protocols; group S 24, 24 hr incubation in rich medium after synchronization protocol.

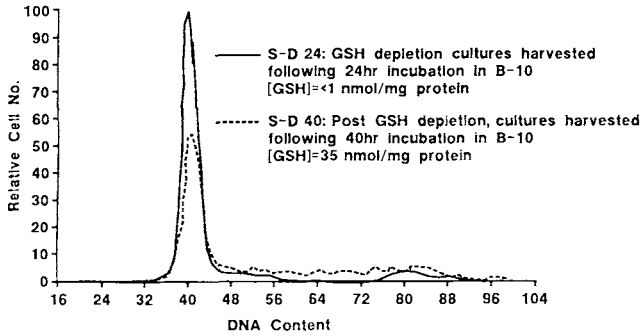

Fig. 3. Normalized data (based on establishment of peak G1 distribution of group D cultures to 100), obtained during concurrently conducted experiments, of DNA related fluorescence of propidium iodide stained human gingival fibroblast nuclei that were harvested following: S-D 24, 24 hr incubation in rich medium post synchronization and GSH depletion; S-D 40, 40 hr incubation in rich medium postsynchronization and GSH depletion.

cent oxidation (29.1%) and total nicotinamide nucleotide levels (9.0 nmol/mg) relative to the S-D 24 cultures, which were harvested at 24 hr.

Results obtained from the flow cytometric DNA analyses showed an association between cellular sustained mitogenic responsiveness and a specific cellular thiol redox state. When cultures progressed beyond G1, they contained abundant reducing equivalents in the form of reduced nicotinamide nucleotides and increased GSH levels. Synchronized cultures had recovered from the ethionine-induced redox poise oxidation after 24 hr in B-10 and progressed beyond G1. In contrast, the S-D 24 cultures, which retained an oxidized redox poise and depleted GSH levels after 24 hr in B-10, did not show cell cycle progression.

Groups S and S-D 24 contained comparable population distributions in the G1, growth-arrested state despite the fact that the S-D 24 cultures had an additional 24 hr in B-10 after synchronization (Fig. 2). In comparison to either the S or the S-D 24 cultures, the S 24 cultures showed a significant difference ($P < 0.001$ Mann Whitney U test) in cell cycle progression. Formerly G1, growth-arrested cultures resumed cell cycle progression following cellular GSH and redox poise recovery (Fig. 3).

Results of the bFGF assays (immediate mitogen responsiveness) revealed that there existed statistically significant differences between our culture groups in cellular capacities to respond to a mitogenic stimulus by increasing $[Ca^{2+}]_i$ (Table II). Prior to the addition of bFGF, there were no significant differences noted in resting $[Ca^{2+}]_i$ among groups L, S, S 24, or S-D 24. This finding demonstrated that cellular resting $[Ca^{2+}]_i$ were not affected by thiol redox perturbation. All culture groups showed a response lag period (between 0 and 30 sec) following addition of bFGF. Maximal increases in $[Ca^{2+}]_i$ were recorded 1–3 min after FGF challenge, after which

TABLE II. Increase in $[Ca^{2+}]_i$ in Response to bFGF†

Experimental group (n)[a]	$[Ca^{2+}]_i$ (nM) pre-b FGF	Percent increase in $[Ca^{2+}]_i$
L intrinsic (5)	213 ± 39	17.8 ± 7.5*
L challenge (4)	360 ± 44	13.0 ± 1.4**
S intrinsic (4)	209 ± 26	8.6 ± 1.4*,***
S challenge (4)	346 ± 40	5.1 ± 3.3**,***
S 24 intrinsic (4)	208 ± 37	17.9 ± 8.2**
S 24 challenge	394 ± 38	17.0 ± 7.5
S-D 24 intrinsic (4)	196 ± 30	7.4 ± 3.1*,***
S-D 24 challenge (4)	350 ± 30	4.7 ± 3.0**,***

†All data expressed as mean % increase above nonchallenged $[Ca^{2+}]_i$ ± S.D., n = number of separate cultures in each assay.

[a]Intrinsic: fura-loaded cells resuspended in PBS; challenge: fura-loaded cells resuspended in Ca^{2+} mobilization buffer.

*$P < 0.008$.

**$P < 0.014$.

***$P < 0.029$.

$[Ca^{2+}]_i$ slowly declined and then plateaued at levels 1.5–2-fold above resting $[Ca^{2+}]_i$. Groups L and S 24 showed the highest bFGF responses, with maximal levels of $[Ca^{2+}]_i$ recorded when these cells were suspended in the "Ca^{2+} mobilization buffer." (Maximal stimulated $[Ca^{2+}]_i$ 406.7 ± 49.5 [SD] nM and 401.0 ± 44.4 [SD] nM, n = 4, for each group, groups L and S 24, respectively.) The Kruskal-Wallis one-way analysis of variance test showed a $P < 0.05$ for both the intrinsic and challenge parameters in comparison of groups L, S, S 24, and S-D 24. Next, a Mann Whitney U test was conducted to determine between which specific groups (L, S, S 24, or S-D 24) the differences existed. The same levels of significance were found (intrinsic $P < 0.008$, challenge $P < 0.014$) in comparison of the log growth (L) cultures with either the synchronized (S) or the synchronized, GSH-depleted cultures (S-D 24) in cellular capacities to mobilize Ca^{2+}. Comparison of the synchronized, 24 hr B-10 (S 24) culture Ca^{2+} results vs. either the S or S-D 24 group results showed that the S 24 cultures responded with significantly higher $[Ca^{2+}]_i$, $P < 0.029$ for both the intrinsic and challenge parameters. No difference in Ca^{2+} mobilization capacity was found in comparison of the L vs. S 24 culture groups.

Comparison of our redox poise, Ca^{2+} mobilization, and cell cycle progression results demonstrated the association of cellular thiol redox state with mitogen-induced responses. After 24 hr in B-10, the redox poise of the S 24 cultures became more reduced, and then both intracellular GSH levels and redox state approximated those in L cultures. The increases in $[Ca^{2+}]_i$ in response to bFGF was also similar in these two groups. In addition, the S 24 cultures showed a sustained mitogenic responsiveness by demonstrating significant cell cycle progression beyond their G1 growth-arrested state after 24 hr in rich medium. In contrast, the redox state of both the S and the S-D 24 cultures remained significantly oxidized at harvest. These culture groups were also less capable of responding to mitogenic stimuli by Ca^{2+} mobilization or cell cycle progression. There exists a strong positive correlation in comparison of culture group mean levels of reduced nicotinamide nucleotides with culture capacity for mitogen-induced Ca^{2+} mobilization (Fig. 4) (r = 0.995 intrinsic vs. mean levels reduced nicotinamide nucleotides, r = 0.938 challenge vs. mean levels reduced nicotinamide nucleotides.)

DISCUSSION

The regulation of cell cycle progression is complex and entails the interaction between extracellular factors and intracellular responses [26,42–44]. Evidence suggests that intracellular factors are key in determining how cells respond to mitogens [26,42–44]. In vivo, cells are bathed in mitogen-rich serum, yet are responsive to these mitogens only during the G1 phase of the cell cycle [26,42–44]. Previous studies have reported G1-related changes in cellular GSH and thiol levels [45–47]. However, these reports did not evaluate the relationship of these constitutents with the position of the cell in the cell cycle. The purpose of this investigation was to evaluate if the cellular thiol redox status is a determinant in how cells in G1 respond to mitogens. The results imply that cellular thiol redox status is a critical intracellular parameter that affects how mitogens and cells in G1 interact.

The cell cycle distribution and redox poise results showed that ethionine performed two experimental functions. First, the ethionine synchronization provided a G1 stage-specific population baseline. Second, exposure to ethionine perturbed the cellular redox poise, which was one of the intracellular parameters that was to be investigated. Ethionine caused an oxidation of the cellular redox state and an increase in cellular total nicotinamide nucleotide levels. These redox poise alterations were noted in cultures harvested immediately after the ethionine

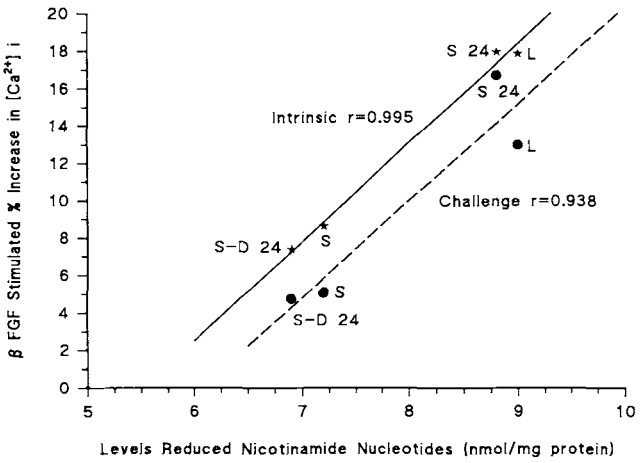

Fig. 4. Relationship between mean levels of reduced nicotinamide nucleotides and bFGF-stimulated percent $[Ca^{2+}]_i$ in human gingival fibroblasts from the following experimental groups: L, log growth; S, synchronized, harvested after the 6 day ethionine synchronization; S 24, synchronized, harvested after an additional 24 hr incubation in B-10; S-D 24, Synchronized, GSH depleted, harvested after an additional 24 hr incubation in B-10; intrinsic, fura-loaded cells suspended in PBS; challenge, fura loaded cells suspended in "Ca^{2+} mobilization buffer" (see Materials and Methods).

synchronization and in synchronized, GSH-depleted (S-D 24) cultures. Both these redox poise changes may be attributable to a bioenergetic perturbation. The intracellular availability of adenine nucleotides would be affected by the presence of ethionine [48]. The increased intracellular oxidation should stimulate reducing equivalent generating enzymes, e.g., isocitrate dehydrogenase, malic enzyme, and the first two enzymes of the hexose monophosphate shunt, thus accounting for the increases in total nicotinamide nucleotides [49]. Because the depletion of cellular GSH removes an important source of reducing equivalents, the S-D cultures faced an additional obstacle en route to thiol redox recovery [27,28]. This was reflected by the delayed recovery from growth arrest in the S-D cultures.

The redox poise findings compared favorably with reported values [50]. Results obtained from the S and S-D 24 cultures agreed well in both total and reduced/oxidized distributions with other "stressed" cells, those harvested from fasted animals [50]. The L and S 24 total nicotinamide nucleotide levels compared well with results obtained from cells harvested during the fed state [50].

The $[Ca^{2+}]_i$ levels obtained during the resting and bFGF challenged assays are in good agreement with previously published reports [18,51,52]. Significant intergroup differences, which were related to the cellular redox state, were detected in the bFGF Ca^{2+} mobilization assays. The decreased Ca^{2+} mobilization observed in the S and S-D 24 groups may reflect a perturbation of the interrelated cellular redox poise and bioenergetic status. During mitogenic signal transduction, many growth factors promote Ca^{2+} mobilization in conjunction with activation of the Na^+/H^+ antiport [53–55]. The adenosine triphosphate (ATP) may not be available to stimulate the monovalent ion fluxes in these bioenergetically perturbed cultures.

Variability in cellular growth factor expression has been cited as a potential cause for differences in $[Ca^{2+}]_i$ responses [51]. Because cellular harvesting and mitogen response assays were conducted in an identical fashion for all groups, any variations in receptor numbers or expression would be due to intrinsic cellular differences. The differences noted in mitogenic responsiveness may indeed reflect a growth factor receptor down-regulation that would result in decreased bFGF binding in those cultures that were less mitogen responsive (groups S and S-D 24).

The bFGF stimulated Ca^{2+} results showed intragroup variability, as depicted by the standard deviations within each group. This study was conducted on human cell strains, not established cell lines. Therefore, variability among individual cell strains would be expected. It is well accepted that cellular populations comprise various subpopulations, which include cells that are heterogeneous in their responses to Ca^{2+} mobilization [18,56]. Via the fura-fluorometric

assay, significant differences were detected among the biochemical averages of the experimental groups. The flow cytometric DNA analyses complimented the fura data by establishing that cellular subpopulations existed in regard to mitogenic responses.

Specific segments of the endoplasmic reticulum, some of which communicate with the plasma membrane, are proposed to provide the Ca^{2+} source for the transient, mitogen-induced, increases in $[Ca^{2+}]_i$ [17]. Previous publications have demonstrated an association between the regulation of Ca^{2+} release from mitochondria and endoplasmic reticulum and the redox state of nicotinamide nucleotides [19,20,57,58]. Oxidation of the redox state promoted a mitochondrial Ca^{2+} efflux [19,20]. In addition, investigators who have reported other cation membrane transport studies have proposed that NAD(P)H, or a group in close redox communication, e.g., a membrane bound thiol, functions to restrict cation flow through membrane channels [21,22]. Furthermore, microsomal Ca^{2+} sequestration has been shown to be perturbed by reagents that promote oxidation or diminish ATP levels [59]. Both of these cellular alterations, depletion of ATP and redox poise oxidation, could result from ethionine exposure [48].

In this study, resting $[Ca^{2+}]_i$ levels were comparable, whereas differences were noted in the "mitogen-responsive" Ca^{2+} pool. Volpe et al. [60] recently proposed that discrete "calciosomes" make up the responsive Ca^{2+} pool in the endoplasmic reticulum. Because Ca^{2+} efflux in mitochondria was associated with an oxidized redox state, we propose two plausible explanations for our Ca^{2+} results. First, prior to mitogen interaction, an inhibition of Ca^{2+} sequestration occurred in the thiol redox-perturbed cultures. The released Ca^{2+} would then be readily sequestered by mitochondria, thereby maintaining normal $[Ca^{2+}]_i$. Alternatively, the Ca^{2+} loading into the responsive pool may not have occurred in those cells with an oxidized redox poise if it is an ATP-dependent process. In either situation, a mitogen-responsive pool of Ca^{2+} would not exist in the thiol redox-perturbed cultures.

This study evaluated two parameters of the mitogenic response. Ca^{2+} mobilization and cell cycle progression were the immediate and sustained parameters, respectively. These mitogenic responses were interrelated with the cellular thiol redox state. Cultures with increased GSH and reduced nicotinamide nucleotide levels showed Ca^{2+} mobilization and also showed cell cycle progression, implying a complete mitogenic signal transduction.

Because cells employ an enzymatic means of rapid response, mitogenic signal transduction is a rapidly occurring event [61]. A cascade of kinases, which serve to transmit and amplify the initial message, is activated during the mitogenic response [26]. Because many of these kinases are Ca^{2+} dependent, kinase cascade stimulation is dependent on an increase in $[Ca^{2+}]_i$ [26]. To transduce the mitogenic signal, a cell must transiently increase $[Ca^{2+}]_i$ to augment the activation of the kinase cascade [26].

In conclusion, we propose that the cellular response to those mitogenic stimuli that function via inositol phosphates is interdependent on cellular thiol redox status. Cells that have a cellular thiol redox status below a threshold response point, may have compromised Ca^{2+} mobilization and therefore may be incapable of triggering the mitogen-induced cascade.

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