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Glutamine prevents DMBA-induced squamous cell cancer

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Summary The etiology of oral squamous cell carcinoma has been linked to environmental carcinogens, such as activated aromatic heterocyclic radicals and epoxides. Our previous work on implantable and 7,12-dimethylbenz[a]anthracene (DMBA)-induced breast cancer showed that oral glutamine (GLN) inhibited tumor growth possibly through stimulation of host – and selective inhibition of tumor glutathione (GSH). This finding was associated with up-regulation of NK cell activity, decreased IGF-1 and TGF-β in the circulation and downregulation of PI-3K/Akt antiapoptotic signaling in tumors. The present study was designed to investigate the effect of topically applied GLN on DMBA-induced hamster buccal pouch squamous cell carcinoma. Histopathological alterations in buccal pouches were studied by light microscopy. GLN and GSH levels in blood and buccal mucosa were determined using specific enzyme assays. The protein expression of bax, bcl-2 and PARP was determined by western blotting. *H-ras* and *p53* genes were examined for presence of mutations using direct DNA sequencing. Fourteen weeks after DMBA application none of the GLN-supplemented animals developed tumors, while all of the control animals had well developed squamous cell carcinomas. The inhibition of DMBA-carcinogenesis by GLN application was associated with increased arterial GLN and GSH, elevated buccal mucosa GSH as well as induction of bax and PARP, and inhibition of bcl-2. *H-ras* and *p53* were wild type. The results from this study in combination with our previous data suggest that

Abbreviations: GLN, glutamine; GSH, glutathione; DMBA, 7,12-dimethylbenz[a]anthracene; BPM, buccal pouch mucosa.

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the chemopreventive effects of GLN are exerted by enhancing the antioxidant status of the body and activation of apoptosis.

Introduction

There is an estimated 19,000 new cases of oral cavity carcinoma diagnosed annually in the United States, and 5000 people die of the disease each year. The oral carcinoma accounts for 4% of all cancers in men and 2% of all cancers in women.[1,2] The etiology of this cancer has been strongly linked to environmental carcinogens. The synergistic link of excessive alcohol and tobacco use and squamous cell carcinoma (SCC) of the head and neck may be responsible for approximately 75% of these tumors.[3] The nature of these chemical carcinogens has not been fully elucidated, but the involvement of oxidative damages and/or genetic mutations have been suggested[4,5] and warrants the use of carcinogen-induced animal models.

Glutamine (GLN) is a non-essential amino acid, that becomes ''conditionally essential'' during periods of catabolic stress, when its use exceeds its production.[6] This characteristic depletion of GLN is attributed to its multiple roles in the body, including roles as a metabolic intermediate contributing carbon and nitrogen for the synthesis of other amino acids, fatty acids, nucleic acids and proteins, supplying a source of fuel for dividing cells[7], and as a substrate for synthesis of the main natural antioxidant glutathione (GSH). GSH (glutamyl—cysteinyl—glycine) is a sulfhydryl (—SH) antioxidant, ubiquitous in animals, plants, and microorganisms. GSH often attains millimolar levels inside cells, which makes it one of the most highly concentrated intracellular antioxidants.[8] The ratio between reduced (GSH) and oxidized (GSSG) glutathione (GSH/GSSG) is considered a sensitive indicator of oxidative stress.[9] Cancer development is associated with marked depletion of skeletal muscle GLN, accompanied by decreased GSH levels.[10] In the presence of oxidative stress, GLN might become a rate-limiting substrate for the synthesis of GSH.[11] Our previous studies showed that oral dietary GLN caused paradoxical lowering of GSH content in tumors, while increasing it in normal tissue.[12—15] This is important, because as a key regulator of the cellular redox state GSH determines the response of tumors (and protection of the normal cells) to chemotherapy and radiation.[16—18] Resistance to a variety of anticancer agents is often associated with increased GSH levels in the tumor cells[19,20] indicating that reduction of tumor GSH may enhance tumor sensitivity to radiation and chemotherapy.

Intracellular GSH/GSSG ratio is crucial for activation of cell proliferation and cell death.[21] Functional studies have determined that the elevated expression of anti-apoptotic-(bcl-2, bcl-XL, mcl-1, A1) or diminished expression of the proapoptotic Bcl-2 family members (bad, bax, bid, bik, bak, bcl-XS) can inhibit the mitochondrial apoptotic pathway (reviewed in [22—24]). Heterodimeric and homodimeric coupling of these various proteins, which ultimately depends on the relative proportion of each protein, determines the eventual direction of the apoptotic mechanism.[25—27] Apoptosis and specifically bcl-2 and bax proteins have been thought to have some prognostic implications in head and neck squamous cell carcinoma.[28] An important factor in induction of apoptosis is poly(ADP-ribose) polymerase (PARP), a target of the main apoptosis-executing enzyme caspase-3. PARP cleavage has now been recognized as a sensitive marker of caspase-mediated apoptosis.[29] One of the mechanisms that may trigger apoptosis is oxidative/reductive (redox) DNA damage.[30]

The aim of the present study is to examine the effect of supplemental topical GLN on the development of DMBA-induced squamous cell carcinoma in the hamster buccal pouch carcinogenesis model.

Materials and methods

Experimental animals and treatment

Sixty male adult Syrian Golden Hamsters (150 g) were obtained from Harlan Sprague-Dawley, Inc., (St. Louis, MO). All studies were approved by the Animal Care and Use Committee at the John L. McClellan Veteran's Hospital. The hamsters were maintained in cages in the animal care facility and were subjected to alternate 12 h periods of dark/light cycle and given at least one week to acclimate to the animal care facilities. During that time the hamsters were allowed ad libitum intake of standard hamster chow and water. During the study period of 14 weeks the animals were pair-fed chow and allowed ad libitum intake of water. After acclimation, the hamsters were randomized to be treated topically with 1 g/kg/day GLN (Sigma Chemical Co, St. Louis, MO) or with 1 g/kg/day of Promod (PRO, protein modules, Ross Laboratories, Columbus, OH). GLN and Promod were placed into the right buccal pouch daily. Control hamsters (CON) were stimulated with a clean metal spatula once daily. Animals were further randomized to receive DMBA in heavy mineral oil as vehicle or only vehicle. A solution of 0.5% DMBA in heavy mineral oil was painted onto the right BPM of the DMBA-groups of hamsters using a #4 sable brush 3 times a week. We applied DMBA at a separate time from the glutamine so as to avoid direct interaction between the two chemically. Each application leaves approximately 0.6 + 0.2 mg of DMBA on the mucosal surface. Control animals (GLN, PRO, CON) were similarly treated with heavy mineral oil. Thus all hamsters were randomized into one of the following 6 groups of 10 animals each: GLN + DMBA, PRO + DMBA, CON + DMBA, GLN, PRO, CON.

Animals were examined weekly for tumor development. Fourteen weeks later the hamsters were anesthetized by intraperitoneal injection of pentobarbital sodium (Nembutal) (50 mg/kg) and sacrificed. Right and left buccal mucosa were inverted and sharply resected. Tumor number, volume, and weight were measured. Tumor diameter was measured using Vernier calipers. Tumor volume was determined

by multiplying the number of tumors in each group by the mean tumor volume in millimeters ($4/3\Sigma r3$, where r represents 1/2 tumor diameter in mm). Tumor tissue was stored in 10% formalin for tumor morphometrics. Blood was obtained with a 25-gauge needle from the aorta which was exposed through a midline incision.

GLN measurement

All chemicals were purchased from Sigma Chemical Co., (St. Louis, MO). Aliquots of heparinized whole blood were mixed with equal volumes of cold 10% perchloric acid, then vortexed and centrifuged at 5 °C at 3000g for 15 min (Sorvall Model RC5). The supernatant was removed and neutralized with an equal amount of cold 0.48 M potassium phosphate (Sigma Chemical Co., St. Louis, MO). This was again vortexed and centrifuged at 5 °C at 3000g for 10 min. The supernatant was removed and kept frozen at −20 °C for later determination of GLN concentration by the microanalytical method described by Bergmeyer and Bernt[31] and Nahorski.[32] For glutamate (GLU) assay, 100 μl above supernatant was added to 1 ml reaction mixture, that contained 0.73 mg β-nicotinamide adenine dinucleotide (β-NAD), 10 μl 0.1 M ethylenediamine tetraacetic acid (EDTA), 1 μl mercaptoethanol in tris buffer, and 10 μl glutamate dehydrogenase (GDH). The reaction mixture was incubated in 37 °C water bath for 30 min, and the fluorescence was measured using fluorometer. To assay for total GLN and GLU, 50 μl of the supernatant was added to 0.5 ml of 0.2 M sodium acetate and 0.025 units glutaminase, incubated in 37 °C shaking water bath for 45 min and the reaction was stopped with 0.5 ml water. This was followed by the above procedure. The GLN and GLU concentrations were determined by a standard curve. The net GLN concentration was calculated by subtracting the GLU concentration from the total GLN + GLU concentration. The results are expressed as μmol/l.

GSH measurement

Total tumor and arterial blood GSH and GSSG were measured via a standard enzymatic recycling method, as described by Tietze[33] and modified by Anderson.[34] To measure tumor GSH, 0.5 g tissue were homogenized with 2.5 ml 5% 5-sulfosalicylic acid (Sigma Chemical Co., St. Louis, MO), the protein content was measured and the samples were centrifuged at 3000*g*, 4 °C for 15 min. The supernatant was kept frozen at −80 °C until used for GSH assay. GSH was measured as follows: 10 μl of the supernatant were added to 1 ml of reaction mix (0.2 mM reduced nicotine amide adenine dinucleotide phosphate, 0.6 mM 5,5-dithiobis-2-nitrobenzoic acid and 1.33 units GSH reductase) and the absorbance was measured at 412 nm. To determine GSH disulfide (GSSG) content, 0.5 ml of the supernatant was mixed with 10 μl 2-vinyl pyridine and 60 μl triethanolamine in order to remove GSH via the method of Griffith[35], and then measured according to the above procedures. The data were normalized by *g* of protein and expressed as μg/g tissue. To measure arterial GSH, aliquots of heparinized whole blood were immediately mixed with equal volumes of 10% 5-sulfosalicyclic acid, vortexed and centrifuged at 3000*g*, 4 °C for 15 min. The supernatant was removed and kept frozen at −80 °C until used for GSH assay as described above. The results were expressed as μmol/l.

Histopathological studies

Specimens for light microscopy were immediately fixed in 10% buffered formalin and subsequently embedded in paraffin media. Several 6 μl tissue sections were cut from each paraffin block and mounted on glass slides. The slides were stained with hematoxylin and eosin. Histological evaluation of the mucosal and tumor specimens was made in a blinded fashion by one pathologist in the Department of Pathology.

SDS—PAGE and immunoblotting

Protein extracts from right BPM of eight animals from each GLN + DMBA and CON + DMBA groups were prepared by homogenization in the following lyses buffer: 10 mM Tris—HCl, pH 7.6/5 mM EDTA/50 mM NaCl/30 mM $Na_4P_2O_7$/50 mM NaF/200 μM Na_3VO_4/1% Triton-X 100 and 1 tablet/50 ml buffer of protease inhibitor cocktail tablets (Roche Diagnostics GmbH, Mannheim, Germany). The protein concentrations of the supernatants were measured using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). Forty micrograms total protein from each sample were fractionated on 10% polyacrylamide gels and transferred onto PVDF membrane. Equal protein loading was controlled by staining the membranes with 0.2% Ponceau S. After blocking with 5% non-fat milk in TBS-T buffer (100 mM Tris, pH 7.5; 150 mM NaCl, 0.1% Tween 20) for 1 h at room temperature, the membranes were incubated overnight at 4 °C with primary antibody, washed in TBS-T and incubated with HRP-labeled secondary antibody for 1 h at room temperature. The primary antibodies, anti-bcl-2, anti-bax and anti-PARP, were purchased from Cell Signaling Technology (Beverly, MA). The secondary antibody, anti-rabbit IgG was from Santa Cruz Biotech (Santa Cruz Biotech., Inc., Santa Cruz, CA). The equal protein loading was verified by re-probing the membranes with anti-β-actin antibody (Santa Cruz Biotech., Inc., Santa Cruz, CA). Proteins were visualized using the ECL Plus Western Blotting Detection System (Amersham Biosci., Piscataway, NJ).

DNA extraction and direct sequencing of p53 and H-ras

Direct DNA sequencing of exon 1 (codons 12 and 13) and exon 2 (codon 61) of *H-ras* gene and exons 5, 7 and 8 (codons 135, 216, 248, 251, 281, 522) of *p53* was performed on 5 samples from right BPM of each DMBA + GLN, DMBA + ProMod and CON + DMBA groups. Genomic DNA was isolated using DNeasy Tissue Kit (Qiagen, Chatsworth, CA). One micrograms DNA was PCR amplified using synthetic hamster-specific oligonucleotide primers, synthesized by Sigma—Genosys (The Woodlands, TX). The sequences of the primers derived from published hamster *H-ras* and *p53* sequences (GenBank Acc# M84166; GenBank Acc# M75144) are presented in Table 1. The amplifications were performed in 50 μl reaction mixtures using Master mix (Qiagen, Chatsworth, CA). The parameters for the amplifications were: initial denaturation at 96 °C for 2 min, followed by

Table 1 Oligonucleotides used for direct sequencing of *p53* and *H-ras*

	Forward primers	Reverse primers
H-ras	AAGCGATGACAGAATACAAGCTC	TGTTAGATCAGGTGAAACAGACTC
	ACCCCTAAGCTCTGTTCTTCTG	CTGATGGATGTCTTCAAAGGACTTG
p53	TCATCAGCTCCAACTCTGACCCTG	CAGCTGCACGGGGCATGTTTTCGC
	TACTCACCTTCCCTCAATAAGCTG	AAGAGCAATCAAGAACATCAACGG
	CTTACTGTCTCGTGCTCTCCCTCC	TGAAGCTGAACCTCCTCCTCTGCC

35 cycles of 94 °C for 45 s, appropriate annealing temperature for 45 s and 1 min at 72 °C. The final extension at 72 °C was for 5 min. The products were run on a 2% low-melting agarose (Sigma—Aldrich, St. Louis, MO) gel, the bands of the expected sizes were excised and the cDNAs were eluted using QIAquick Gel Extraction Kit (Qiagen, Chatsworth, CA). The sequencing in both directions was performed in DNA Sequencing Core Facility of UAMS, Little Rock, AR. The sequences were compared with the published sequences for hamster *H-ras* and hamster *p53*, using GCG Wisconsin Package.

Calculations/statistical analysis

All data were expressed as mean ± standard error. Differences between means are considered significant at the $p < 0.05$ level using analysis of variance (ANOVA) and non-paired two tailed *t*-test. Statistical analyses were performed using StatView II.

Results

Effect of GLN on tumor development and histopathology

During the 14 week study period none of the GLN + DMBA animals developed tumors of the DMBA-treated right buccal mucosa. In contrast, all animals in the PRO + DMBA ($n = 10$) and CON + DMBA ($n = 10$) developed tumors of the DMBA-treated right buccal pouch. Figure 1 demonstrates the onset of tumorigenesis in each group. The PRO + DMBA showed a slight delay in tumorigenesis which by week 14 was not different. There was no difference in the number of tumors per animal between the PRO + DMBA and CON + DMBA groups (1.7 ± 0.3 in PRO + DMBA and 1.7 ± 0.2 in CON + DMBA; p, no significant difference). This calculation excluded one animal in the CON + DMBA group which seemed to have a field effect with several tumors. There also was no significant difference in tumor volume (184.5 ± 176.43 mm3 in PRO + DMBA vs. 22.8 ± 7.6 mm3 in CON + DMBA). This calculation included one tumor in the PRO + DMBA group which had a volume of 1771.9 mm3. Excluding this tumor the calculations showed an average volume for the PRO + DMBA group of 8.1 ± 5.3 mm3, $p = ns$. Representative histological samples are presented in Figure 2, including normal appearing right buccal mucosa (away from gross tumor) with mild chronic irritation and hyperkeratosis from GLN + DMBA group (Fig. 2a), dysplasia, nuclear enlargement and inflammation from PRO + DMBA group (Fig. 2b) and carcinoma in situ from CON + DMBA (2c).

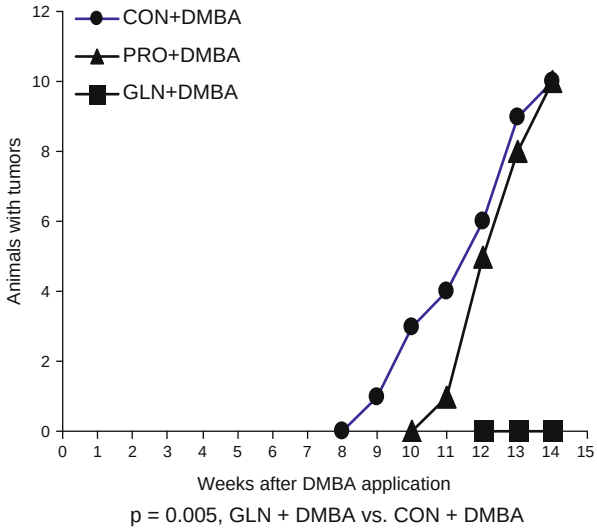

Figure 1 Graphic illustration of tumor appearance per animal in GLN + DMBA, PRO + DMBA, CON + DMBA groups. During the 14 week study period none of the GLN + DMBA animals developed tumors. In contrast, all animals in the PRO + DMBA and CON + DMBA developed tumors.

GLN and GSH levels

The arterial whole blood GLN and GSH concentrations were elevated in GLN-supplemented animals by approximately 20% over PRO and non-supplemented animals (Table 2). DMBA itself did not affect arterial GLN or GSH levels.

There was no difference in GSH tissue levels in similarly supplemented animals regardless of treatment with DMBA or mineral oil control. In the GLN-treated animals the level of reduced tissue GSH of the right buccal mucosa (the supplemented pouch) was elevated by approximately four fold over PRO and non-supplemented animals (Fig. 3A). Reduced GSH levels in the left buccal mucosa (the non-supplemented pouch) were elevated (about 50%) as compared to the PRO and non-supplemented animals, but to a lesser extent than that of the right buccal mucosa (Fig. 3B). Oxidized GSH (not shown) was not significantly different between the groups.

Effect of GLN on protein levels of bax, bcl-2 and PARP

The product of proto-oncogene bcl-2 is known to play a role in promoting cell survival and inhibition of apoptosis, while bax, a member of the bcl-2 family in induction of apoptosis.

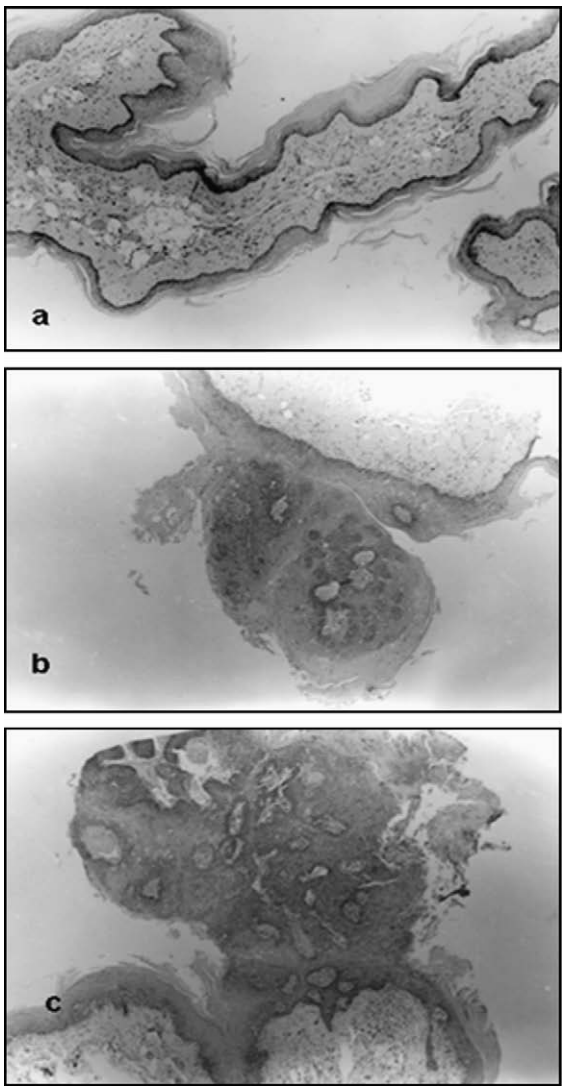

Figure 2 Representative histological samples. (a) Normal appearing mucosa of animal from GLN + DMBA group, showing mild inflammation, hyperkeratosis and parakeratosis (200× magnification); (b) mucosa away from the visual gross tumor showing dysplasia, incomplete maturation, nuclear enlargement and associated inflammation (200×); (c) mucosa away from the visually gross tumor showing carcinoma in situ (200× magnification).

Table 2 Arterial GLN and GSH

	Arterial GLN (μmol/l)	Arterial GSH** (μmol/l)
GLN + DMBA	666 ± 39*	588 ± 38*
PRO + DMBA	563 ± 34	479 ± 24
CON + DMBA	558 ± 31	483 ± 18
GLN	688 ± 43*	519 ± 19*
PRO	583 ± 35	448 ± 19
CON	557 ± 33	484 ± 16

* $P < 0.05$, compared with GLN-supplemented groups, ANOVA.
** Reduced GSH.

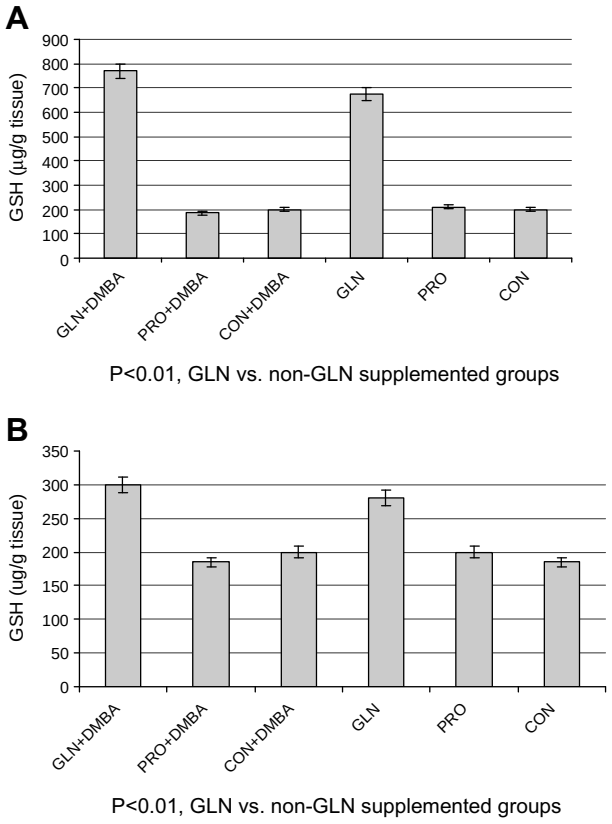

Figure 3 Right (A) and left (B) buccal mucosa GSH content (μg/g tissue). In the GLN-treated animals the level of right (GLN-supplemented pouch) buccal mucosa GSH was elevated by approximately four fold versus PRO and non-supplemented animals (A). GSH levels in the left buccal mucosa (the non-supplemented pouch) were elevated (about 50%) as compared to the PRO and non-supplemented animals, but to a lesser extent than that of the right buccal mucosa (B).

Bax and bcl-2 are membrane-bound pore-forming proteins that interact through heterodimerization. Together they regulate the mitochondrial transmembrane passage of cytochrome c, which in turn activates caspase proteins.[25] PARP is a 116 kDa nuclear enzyme that detects and binds DNA strand breaks produced by various genotoxic agents. During apoptosis caspase-3 cleaves PARP into two fragments an 89 kDa fragment containing the catalytic and automodification domains and a 24 KDa fragment containing the DNA binding domain. The 24 kDa product of PARP cleavage contributes to the irreversibility of apoptosis by blocking the access of DNA repair enzymes to DNA strand breaks.[36]

Using western blotting analysis, we examined the effect of GLN on protein expression of bcl-2 and bax, and PARP in the right BPM of rats from GLN + DMBA and CON + DMBA groups. The results presented in Figure 4 showed that GLN application resulted in downregulation of bcl-2, up-regulation of bax and increased cleavage of PARP.

DNA sequencing

In the experimental studies of DMBA-induced squamous cell carcinomas in hamster buccal pouch epithelium Chang et al.[37]

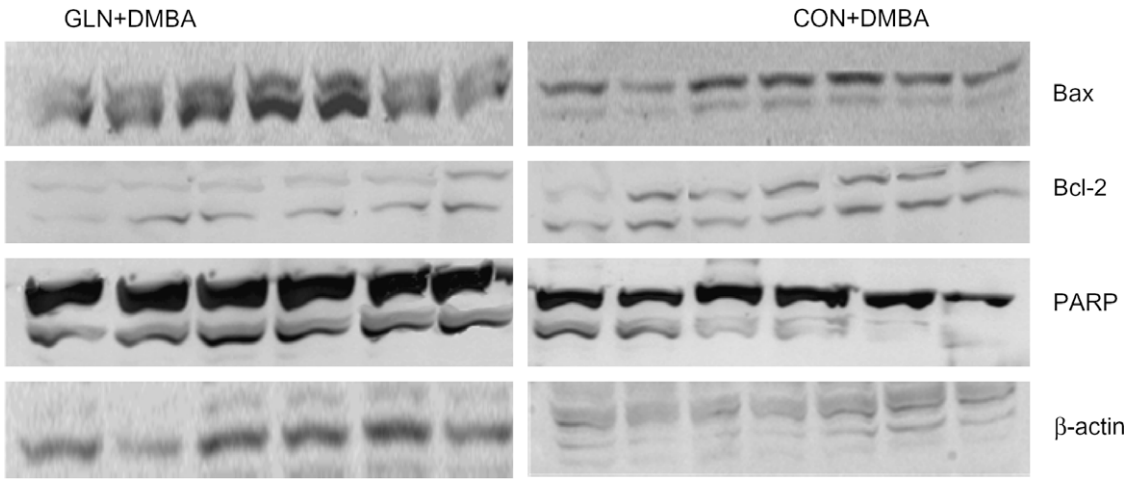

Figure 4 Protein expression of bax, bcl-2 and PARP. Equal protein loading was controlled by staining the membranes with 0.2% Ponceau S (not shown) and reprobing the membranes with β-actin.

established mutations in 25% of the carcinomas in codon 248 of p53 and in 37% of the carcinomas in codon 61of *H-ras*. Gimenez-Conti et al.[38] examined DMBA-induced squamous cell carcinomas in hamster buccal pouch epithelium and found mutations in *p53* in 10% of the tumors, including 1 mutation in codon 216 (exon 6), 2 mutations in codons 251 and 252 (exon 7), and 1 mutation in codon 282 (exon 8). Mutations on codons 12 and 13 of the 2nd exon of *H-ras* (Ref. [39]) and several codons of the conserved exons 5—8 of *p53* were found in a number of human cancers, including head and neck cancer.[40,41]

In this study we have sequenced exon 1 (codons 12 and 13) and exon 2 (codon 61) of *H-ras* gene and exons 5, 7 and 8 (codons 135, 216, 248, 251, 281, 522) of *p53* using direct sequencing of PRC amplified gDNA isolated from the right BPM of 5 hamsters from each GLN + DMBA and CON + DMBA. The results showed that both *H-ras* and *p53* were of wild type.

Discussion

The Syrian golden hamster buccal pouch carcinogenesis model closely mimics events in the development of precancerous lesions and epidermoid carcinomas of the oral cavity in humans using topically and chronically applied DMBA.[42,43] This model produces 100% squamous cell carcinoma of the buccal pouch mucosa in 12—14 weeks.[43]

DMBA is a member of the polycyclic aromatic hydrocarbons that are present in the environment as products of incomplete combustion of complex hydrocarbons.[44—46] Being an indirect carcinogen, DMBA requires metabolic activation by cytochrome P450 to form diol epoxide and other reactive oxygen species (ROS) that are known to increase intracellular oxidation.[47] Therefore administration of antioxidants may retard this process. Antioxidant inhibition of carcinogenesis has been demonstrated with the topical application of alpha-tocopherol[48], beta-carotene[49], oral administration of polyphenolic compounds[50], *N*-acetylcysteine[51], black tea[52] and GSH itself.[53] However, there is no published report of complete inhibition of carcinogenesis in the DMBA model.

GLN is the most abundant amino acid in the blood, comprising more than 60% of the whole body pool of free amino acids. GLN serves multiple metabolic functions in the body, such as energy source, principal carrier of nitrogen, substrate for the synthesis of proteins, a precursor for in the synthesis of GSH (Ref. [54]); and this determines its extremely high turnover rate. The significance of GLN in maintaining the body metabolic homeostasis becomes evident during periods of stress, when it becomes a conditionally essential amino acid.[6] Under normal conditions cysteine is the rate-limiting step in GSH production. However, in rat kidney under oxidative stress, Welbourne found that GLN becomes rate-limiting for GSH synthesis (Ref. [11]). Rouse et al.[55] have demonstrated GLN-mediated recovery of depressed levels of GSH in lung, liver, kidney, heart, gut, and muscle after radiation and/or chemotherapy. Furthermore, the results from our previous studies indicated that GLN supplementation stimulated the host GSH production, accompanied by decreased intratumoral GSH levels in an implantable sarcoma model.[56,57] Further studies with DMBA-induced breast cancer have shown that GLN supplementation reduced tumor growth and reversed the depressed GSH levels in normal tissue.[58] This was associated with up-regulation of the decreased NK cell activity[59] suppression of PGE2[60], decreased levels of IGF-1 and TGF-β in the circulation[61], downregulation of PI3K/Akt antiapoptotic signaling; up-regulation of tumor *p53* signaling and down-regulation of c-myc.[62] GLN supplementation has been shown to protect normal mucosal from acute and chronic radiation injury.[63,64]

The aim of the present study was to evaluate the effect of topical application of GLN on DMBA-carcinogenesis in hamster buccal pouch. The results showed that GLN completely inhibited the development of DMBA-induced leukoplakia and epidermoid carcinoma. Isonitrogenous supplementation of protein (PRO-DMBA) showed a slight but non-significant delay in tumorigenesis compared to CON-DMBA, but by week 14 groups were equal in tumor number and volume. Tumor inhibition was associated with a 4-fold increase in buccal mucosal GSH. Elevated to a lesser extent was the opposing cheek pouch (~50%) and the arterial GSH concentrations

(~20%). These elevations in GSH probably represent intrapouch transfer and systemic ingestion, respectively. Interestingly there was no difference in GSH level in the blood or mucosa of PRO- or CON-fed groups whether the animals received DMBA or not. This is in sharp contrast to the findings of Zhang et al.[65] who demonstrated increased GSH in the mucosa of DMBA-treated versus control hamster pouches. The decreased tumorigenesis seen in the present study is consistent with the studies of Rotstein and Slaga[66] who demonstrated decreased squamous cell cancer of the skin with topically applied GSH. The increased levels of GSH resulting from GLN application in this study was associated with downregulation of bcl-2, upregulated bax and increased PARP cleavage. The increased cleavage of PARP suggests promotion of apoptosis in the tissue that was treated with DMBA.

Altogether, the results from the present study in combination with our previous data provide another evidence for the beneficial effects of GLN application (both topical and oral) in carcinogenesis. Moreover, the safety of enteral and parenteral GLN, and its benefits on improving amino acid metabolism, immune function, and outcome in normal volunteers and patients with catabolic diseases have been established in numerous studies, including at least 18 clinical trials (reviewed in [67]). A Phase II study published by our group, demonstrated the safety and efficacy of oral GLN in escalating doses of methotrexate.[68] With its lack of toxicity and low cost GLN can be efficient adjunct to the main anti-cancer therapy.

Conflict of Interest Statements

None declared.

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