Keratinocyte Transglutaminase Expression Varies in Squamous Cell Carcinomas

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Type I transglutaminase (TGase I, keratinocyte or particulate transglutaminase) is a 92-kilodalton (kDa) protein expressed in abundance in cultured keratinocytes and in the hyperproliferative skin disorder psoriasis. To determine the expression of TGase I protein and mRNA, we studied tissue and established squamous carcinoma lines derived from different sources. Immunohistochemistry and Western blotting were used to detect TGase I protein with the B.C1 mouse monoclonal antibody. Only well-differentiated, skin-derived squamous carcinomas stained for TGase I. However, a precocious pattern of expression was seen overlying less-differentiated tumors. Compared to cultured human keratinocytes, squamous cell carcinoma (SCC) had many times less to 7.8 times more TGase I protein, greatest in the two most differentiated tumor lines 14-83 and ME-180. TGase I mRNA levels ranged from 0.010 to 0.00004 pg/μg total RNA by reverse transcriptase-polymerase chain reaction using an internal standard. Protein expression correlated with mRNA levels in most SCC lines. When a human TGase I promoter was isolated and used to study genomic DNA, SCC 1-83 was shown to have unique restriction enzyme fragments, including one indicative of methylation differences, also present within DNA from the KB line. These studies suggest that transcriptional control of TGase I gene expression in squamous carcinomas may be influenced both by *cis* elements in the promoter and by the degree of tumor squamous differentiation. *Key words: epidermal differentiation/epidermis/skin cancer/cornified envelope. J Invest Dermatol 102:462–469, 1994*

Keratinocytes undergoing terminal differentiation form a cornified barrier, the stratum corneum, which is critical for host survival. Skin cancers derived from keratinocytes are the most common of all human cancers, and include basal cell carcinomas (BCCs) and squamous cell carcinomas (SCCs). Squamous cell carcinomas, especially those derived from mucosal surfaces, are more aggressive, metastasize more frequently, and are associated with greater morbidity and mortality than BCCs.

Transglutaminase enzymes catalyze the cross-linking of proteins through formation of ϵ-(γ-glutamyl) lysine isopeptide bonds that help to form the cornified layer [1]. A number of proteins expressed in terminal differentiation are substrates for transglutaminase, including loricrin, involucrin, keratohyalin, and a 130-kDa protein [2,3]. The skin contains three distinct transglutaminases: TGase I (keratinocyte or particulate transglutaminase [4,5]), TGase III (epidermal transglutaminase [6,7]), and TGase II (tissue transglutaminase [8,9]). TGase I, first isolated as a 92-kDa membrane-associated membrane, is recognized by the monoclonal antibody B.C1 [4,5]. TGase I is highly expressed in cultured keratinocytes, is downregulated by administration of retinoic acid, and is induced by TPA, calcium, and cell confluency *in vitro* [10–13].

Psoriasis and squamous cell carcinomas have in common epidermal hyperproliferation and response to retinoids [14]. Psoriasis is an inherited, self-limited reversible state of local epidermal hyperproliferation without progression to malignancy, which may be initiated by inflammation [15]. Precocious expression of TGase I and involucrin has been noted in psoriatic lesions [5,16]. There is a sixfold or greater increase in TGase I mRNA expression, which might explain the hyperkeratosis characteristic of psoriatic lesions [17].

Squamous cell carcinomas arise on any epithelial surface and vary in degree of squamous differentiation. TGase I has been suggested to be a marker for the state of differentiation in lung cancers [18] and in rabbit trachea [19]. In epidermal dysplasia of the oral cavity it has been shown to be uncoupled with involucrin expression [20]. TGase I activity and expression in response to retinoic acid varies among various squamous cell carcinoma lines but the mechanisms are unknown [20–26]. We determined the level of expression of TGase I protein and mRNA in squamous cell carcinomas and observed variation that correlated with the degree of squamous differentiation.

MATERIALS AND METHODS

Immunoperoxidase Detection Seven squamous cell carcinomas, 14 basal cell carcinomas, and 15 normal skin biopsies were embedded and snap-frozen in Tissue-Tek OCT (Mile, Naperville, IN). The SCC tumors, examined by routine histology, included one SCC *in situ*, one keratoacanthoma (considered by some to be a well-differentiated SCC), two well-differentiated SCCs, and three poorly differentiated SCCs. Four-micron sections placed on Poly-L-lysine (Sigma, St. Louis, MO)-coated slides were fixed in

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Abbreviations: BCE, basal cell epithelioma; HUK, human epidermal foreskin keratinocytes; KTG, keratinocyte transglutaminase; RT-PCR, reverse transcriptase-polymerase chain reaction; TGase, transglutaminase.

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cold acetone and incubated overnight with B.C1 at 1 : 4 dilution [4]. TGase I detection was done with goat anti-mouse biotinylated immunoglobulin G (IgG) and streptavidin-biotin immunoperoxidase (LSAB kit, DAKO, Carpinteria, CA) with diaminobenzidine tetrahydrochloride (DAB; DAKO) chromogen, counterstained with 1% Mayer's hematoxylin. Pre-immune mouse sera were used as a control. The slides were examined by light microscopy and photographed.

Normal and Squamous Cell Lines Keratinocytes and fibroblasts were explanted from discarded neonatal foreskin as previously described [27]. Well-characterized squamous carcinomas included 14-83, a very well differentiated SCC derived from the retromolar trigone area, and 1-83, a poorly differentiated aggressive SCC derived from tonsil [28,29]. The two lines were established at the M.D. Anderson Cancer Center. SRB-1 was a moderately differentiated, deeply invasive SCC arising on sun-exposed scalp, which quickly reoccurred, causing fatal intracranial metastases. SRB-12 was a nodal metastasis from a well-differentiated, deeply invasive squamous cell carcinoma of skin of the neck. The KB line (an undifferentiated SCC derived from the floor of the mouth), the A431 line (cervical SCC) [30], and the ME-180 line (vulvar SCC) were purchased from the ATCC, Rockville, Maryland [31]. All lines were found to be free from mycoplasma contamination (Genzyme, Pierce).

Cell Culture Human foreskin keratinocytes were cultured by the modified method of Kitano and Okada as described [27] in keratinocyte growth medium (KGM) serum-free media, 0.15 mM calcium (Clonetics, CA). Explanted human foreskin fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) with 10% fetal bovine serum (FBS) [32]. The following media supplemented with 10% FBS and 1% fungibact (Gibco-BRL) were used to grow SCC lines: DMEM (A431, 1-83), minimum essential medium (MEM) (KB and 14-83), and DMEM/HAM12 (SRB-1 and SRB-12). A431, 14-83, and 183 cells at densities ranging from 3×10^5 to 3×10^6 cell/well were also plated onto chamber well slides (Nunc) in DMEM and Ham's F-12 3 : 1 (FAD) or DMEM media and stained for immunoreactivity.

Skin Equivalent System Cultured fibroblasts (2×10^5 cells) were mixed with 3.5 mg/ml type I collagen (Collaborative Biochemical Products, Bedford, MA) neutralized with 1 N NaOH, and plated in 24-well plates (Costar) at 24 h with keratinocytes or A431 cells (5×10^5 cells per well). Twenty-four hours later gels were raised to the air-liquid interface [33]. FAD medium supplemented with 10% FBS, 400 ng/ml hydrocortisone, 10^{-10} M cholera toxin (Sigma), 10 ng/ml epidermal growth factor, 1% fungibact, 0.089 mM adenine, 0.105 iu/ml insulin was added to feed cells from below, 3 times per week. The rafts were harvested on day 21 and sectioned and stained for immunohistochemistry.

Western Blotting Proteins were extracted as described previously [34] and concentration was determined by the DC Protein Assay (Biorad, Hercules, CA). Five micrograms/lane of protein were electrophoresed over a 10% polyacrylamide gel for 1 h at 200 volts, and electroblotted onto Hybond membranes (Amersham, UK) [35]. Human TGase I was detected using B.C1 at 1 : 100 dilution [5] with mouse monoclonal IgM and IgG (Sigma) as controls. Antibodies were detected using goat-anti-mouse IgG or IgM with ECL-Western chemiluminescence detection on Hyperfilm exposed for 15 or 60 seconds (Amersham) [36]. The 92-kDa TGase I signals were analyzed from the autoradiographs by photoimage densitometry (UVP, Inc). Experiments were performed twice.

Isolation of Total RNA Total RNA was extracted from cultured cells by lysis with RNAzol B (Cinna/Biotecx Laboratories, Houston, TX) and precipitated as described previously [37]. The total RNA concentrations in the supernatants were determined by ultraviolet (UV) spectroscopy at 260 nm.

Quantitation of mRNA by Northern Blotting Equal amounts of RNA (7 μg/lane) electrophoresed on 1.2% formaldehyde agarose gels [38] were transferred to nylon membranes as described [37]. RNA was cross-linked by a 30-second exposure to 254 nm light with a UV-transilluminator (UVP, Inc), detected by UV shadowing [17,39], and photographed with Polaroid 55 film and a Wratten 23 filter. Prehybridization and hybridization were carried out with radioactive oligo-labeled 0.66-kilobase pair (kb) 3′ *Eco*R1 cDNA insert for human TGase I as described previously [17]. Blots were washed twice at room temperature for 30 minutes each with $2 \times$ SSC/0.1% sodium dodecylsulfate (SDS) and $0.1 \times$ SSC/0.1% SDS, and then at 60°C in $0.1 \times$ SSC/0.1% SDS. Blots were autoradiographed against Kodak X-AR film for 14–21 d. These experiments were performed four times.

Quantitation of Specific RNA by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) To develop a more sensitive quantitative assay for TGase I mRNA, an internal standard for TGase I PCR was constructed by inserting an additional 76-base pair (bp) DNA *Rsa*I lambda phage fragment into the cDNA [17] to give KTGÕ. The mutant plasmid was then digested with *Eco*R1 to release a 736-bp fragment used as a competitive template. cDNA was synthesized in a reaction containing 2 μg of total RNA, 5 mM deoxynucleotide triphosphates (dNTPs), 2.5 μM random hexamers, 1 unit RNase inhibitor, and 2.5 units of MoMuLV reverse transcriptase and $1 \times$ PCR buffer II (RNA-PCR kit, Perkins-Elmer-Cetus, Norwalk, CT). One-eighth of the reaction was then coamplified with defined amounts (0.005–0.0005 pg) of competitive template in a 25-μl PCR containing 0.1 μM each specific primer, $1 \times$ PCR buffer II, 0.5 units Amplitaq DNA polymerase, 2 mM $MgCl_2$, and no additional dNTPs, concentration <200 μM [40]. Amplification primers for human TGase I partial 3′ cDNA were KTG82 5′-AAGGAGACCAAGAAGG-3′ and KTG81 5′-CTGTAACCCAGAGCCTTC 3′. Following an initial denaturization at 94°C for 1 min, samples were cycled through denaturing at 94°C × 30 seconds, annealing at 56°C × 30 seconds, and extension at 72°C for 30 seconds for 35 cycles.

The RT-PCR products of endogenous RNA (310 bp) and competitor standard (386 bp) were visualized by UV on 4% Nuseive agarose gels and photographed. Absolute areas of the peak intensities were measured by densitometry on a UVP, Inc. photodocumentation system. The ratios of the competitor band to the endogenous band for each reaction were plotted against the amount of competitor template added for each reaction, and linear regressions were calculated. The specific amount of endogenous TGase I mRNA (expressed in picograms/250 μg total RNA used for RT-PCR) was determined from the point at which the ratio of the intensities equalled 1.0 and was multiplied by 4 to express the data per μg of total RNA.

Southern Blotting of the TGase I Gene To study the structure of the TGase I gene promoter in SCCs, a human genomic TGase I clone was isolated from a human liver genomic library in Lambda Fix using the 1.5-kb *Eco*R1 cDNA insert [17]. One phage contained a 15-kb insert from which a 2.3-kb *Hind*III fragment was 100% identical to a published TGase I promoter beginning at position −382 of previously published promoter sequences and extending 5′ [41].

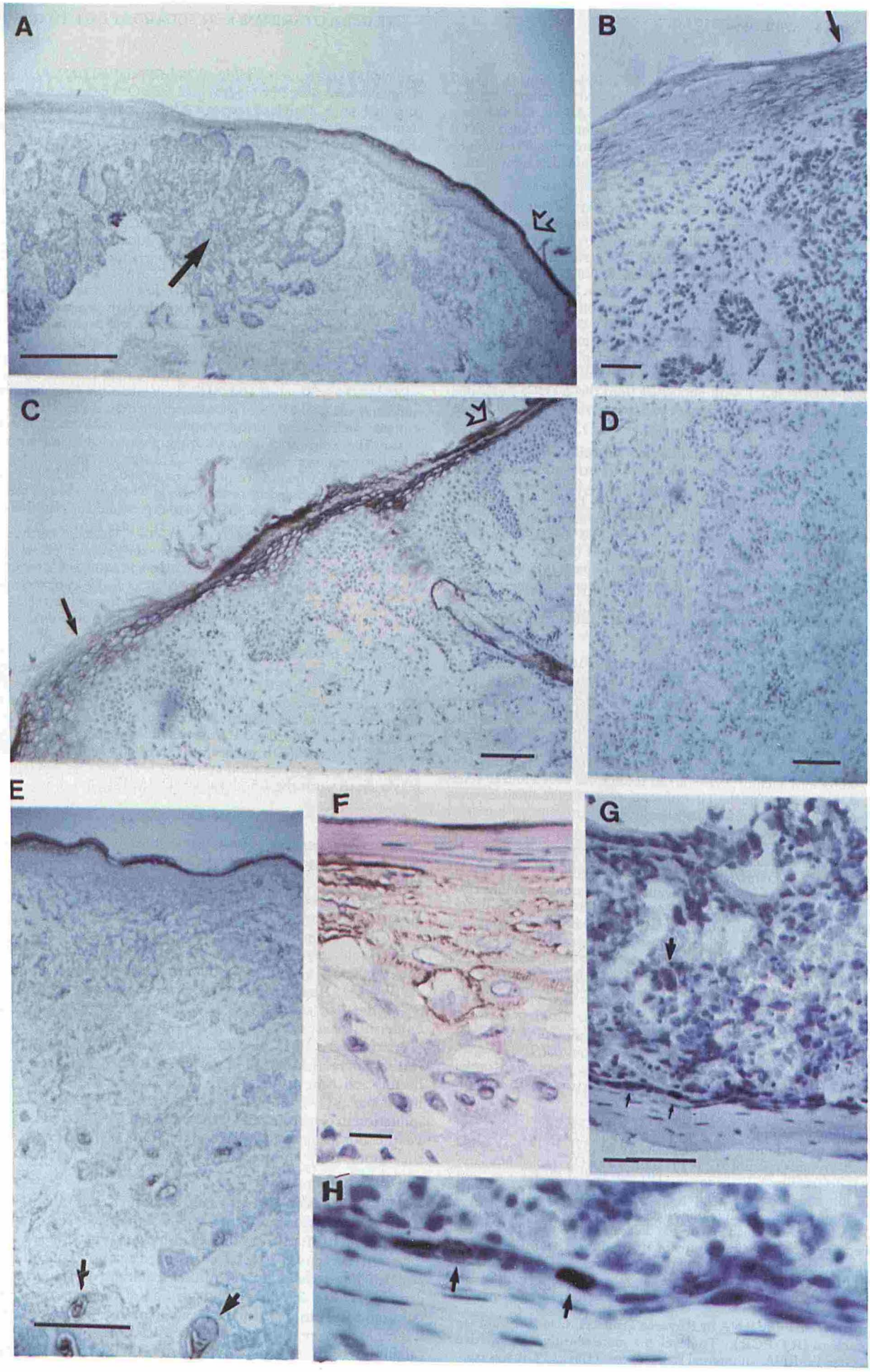
Genomic DNAs (10 μg) extracted from cultured SCC lines (Stratagene kit) [42] were digested by 5 units/μg each of *Eco*RI, *Hind*III, *Msp*I, *Hpa*II, and *Pst*I (Promega). The DNA fragments were separated by 0.8% or 1.2% agarose gel electrophoresis in TEA buffer with lambda *Hind*III and Phi X174 fragments as standards (BRL). Zetabind membranes with transferred DNA were hybridized to ^{32}P-CTP (Amersham)-labeled 2.3-kb *Hind*III genomic TGase I fragment [43], washed, and autoradiographed to detect genomic DNA fragments, as described previously [44]. Blots were stripped [45] and rehybridized with the 1.5-kb partial cDNA [17].

RESULTS

Skin-derived basal cell epitheliomas and invasive squamous carcinomas lack TGase I but induce its expression in overlying normal epidermis.

TGase I expression in patient skin tumors was studied in SCC and BCC tumors and adjacent normal skin using immunohistochemistry. Although basal cell carcinomas did not stain for TGase I (Fig 1*A*), the epidermis overlying these tumors had reactivity extending into the basal layer in the absence of overlying epidermal hyperplasia (Fig 1*B*). Abnormal keratinocytes in invasive, less-differentiated SCCs did not stain (Fig 1*C,D*). However, the hyperproliferative epidermis directly overlying less-differentiated SCC tumors expressed TGase I in a pattern analogous to that observed previously for involucrin [46,47]. The pattern was normal in the adjacent epidermis and hair follicles (Fig 1*C*). Two well-differentiated SCCs had detectable TGase I present in the horn cyst (Fig 1*E*) without induction in overlying epidermis. This suggests that whereas TGase I expression may be lost or absent in SCCs *in vivo*, factors secreted by the tumor may induce enzyme expression in normal, overlying epidermis.

Variable TGase I Protein Expression in SCC Lines Detected by Immunochemistry TGase I was prominent as a 92-kDa band in cultured human foreskin keratinocytes (Fig 2, *lane 2;* Table I). The signal intensity was 1.7 normalized against a control band. The ME-180 line, which is well differentiated and derived from a vulvar carcinoma (Fig 2, *lane 4*), had a band 7.8 times more intense than cultured foreskin keratinocytes, whereas the 14-83 SCC signal was 2.8 times greater (Fig 2, *lane 6*). The SCC lines were grown in high calcium, which is known to induce TGase I, whereas the epidermal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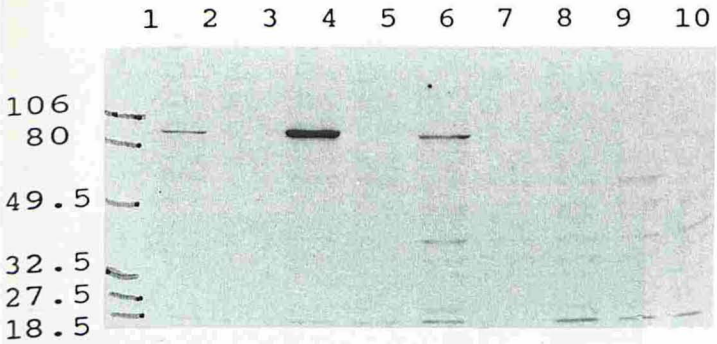

Figure 2. Western blot analysis of TGase I protein in normal and squamous cell carcinoma lines. Five micrograms protein/lane were electrophoresed on 10% SDS-PAGE, electroblotted, and detected with B.C1 MoAb and chemiluminescence (ECLS, Amersham), 15-second exposure. TGase I appears as a 92-kDa band. *Lane 1,* BRL low–molecular-weight markers: 106, 80, 49.5, 32.5, 27.5, and 18.5 kDa; *lane 2,* cultured human foreskin keratinocytes; *lane 3,* adult human fibroblasts from breast reduction; *lane 4,* ME-180 vulvar squamous carcinoma line; *lane 5,* 1-83 tonsil SCC line; *lane 6,* 14-83 trigone SCC line; *lane 7,* SRB-1 SCC line from scalp; *lane 8,* SRB-12 SCC line from back; *lane 9,* KB SCC line from floor of mouth; and *lane 10,* A431 SCC line from genital tract.

keratinocytes were grown in low calcium. Faint 92-kDa bands, several times less intense than in keratinocytes, were seen in the SRB-12 and A431 lanes. TGase I was not detected in the 1-83 or KB lanes or, as expected, in control adult fibroblasts. No signal was detected using secondary IgG antibody alone (not shown).

We studied several SCC lines plated at various densities and at different levels of confluency on chamber slides. The 14-83 line [28] is well differentiated and forms multilayer spheroids that stain heavily for TGase I (not shown). In contrast, 1-83 cells were negative by immunohistochemistry at all plating densities. However, A431 cell expression could be detected in a few cells and was greatly increased at confluency (not shown).

To determine the pattern of expression in A431 cells, we used a skin equivalent system. TGase I staining in normal keratinocytes from foreskins (Fig 1*F*) resembled the pattern seen in psoriasis [16] and overlying SCCs and BCCs. A431 cells, plated on the same dermal equivalent, were mostly negative (Fig 1*G*), with the exception of a few cells found along the epidermal-dermal interface (Fig 1*H*) and rare single cells in the upper layers of the raft (Fig 1*G, arrow*). TGase I did not appear to be readily inducible in A431 cells.

Expression of TGase I mRNA by Northern Blotting Northern blotting (Fig 3*A*) was performed in parallel with Western blotting in SCCs and control lines and loads were assessed by UV shadowing (Fig 3*B*). The signal for most of the mRNA bands was too faint for betagen scope analysis [48], and therefore could not be quantitated directly. Human foreskin keratinocyte mRNA levels were similar to the two SCC lines 14-83 and ME-180. However, the ME180 level of expression was probably higher, considering that the 28S band was less detectable than the other cell lines. A faint mRNA signal was detectable in SRB12 (Fig 3, *Lane 3*), which had a faint protein band. The KB and A431 lines (Fig 3, *Lanes 1, 2*), which did not appear to have protein by Western blotting, had almost no mRNA TGase I signal. However, SRB1 had an mRNA signal with almost no protein signal. Fibroblasts and the 1-83 metastatic SCC line appeared to have neither detectable TGase I mRNA nor protein (Table I). Although the correlation between protein and mRNA expression in most cell lines studied suggests that mRNA transcription may be important in regulating TGase I in squamous carcinomas, other mechanisms may also be operating because some discrepancies appear to exist.

Quantitative RT-PCR Determination of TGase I mRNA RT-PCR was a more sensitive method for the detection of mRNA in SCC lines. An mRNA signal of expected size amplified by RT-PCR was present in all SCC lines. Only dermal adult and neonatal fibroblasts had no mRNA detectable (Table I). Exact equivalency curves plotted from serial titration of competitors were established for cDNAs from keratinocytes and three selected SCC lines. Agarose gels of competitive RT-PCR reactions are shown in Fig 4. The equivalency points of endogenous mRNA versus known standard in pg/μg total RNA are shown in Table I. The endogenous mRNA, a 386-bp product (Fig 4, *right, small top arrow*), was compared to known amounts of competitor 310-bp product (Fig 4, *right, bottom arrow*), amplified with the same PCR primer set. As expected, foreskin keratinocytes, ME-180, and 14-83 cells had greatest expression of the mRNA, in agreement with the Western and Northern blotting data. The mRNA levels of keratinocytes (Fig 4*A*), ME-180 cells (not shown), and SCC 14-83 (Fig 4*C*) were about equal. A431 mRNA (Fig 4*D*) and 1-83 mRNA levels (Fig 4*B*) were 23 and 37 times less than keratinocytes, respectively. The 1-83 RT-PCR reaction also generated two bands (Fig 4*B, open arrow*) that were smaller than the expected endogenous signal of 310 bp. These could result from cDNAs for mutant TGase I alleles or differential splicing, PCR artifacts, or amplification of other genes or random cDNAs.

Alterations in the TGase I Gene are Detected by Southern Blotting in the KB and 1-83 SCC Lines Transcription of mRNA may be controlled at many different levels. Cis factors are DNA sequences in the promoters whereas trans-regulatory factors may include proteins or nucleic acids that bind to specific DNA sequences in the promoter. Therefore, we isolated 5′ genomic sequences within a 2.3 *Hind*III fragment from human TGase I to study the structure of genomic DNA from SCC lines. TGase I promoter and gene fragments were detected by Southern blotting, suggesting that all SCCs studied retain all or most of the TGase I gene (Fig 5). DNA from the 1-83 line gave unique restriction enzyme fragments with several enzymes within the promoter (Fig 5*A*) and the gene (Fig 5*B*). Within the promoter, the 1-83 DNA (Fig 5, *lane 1*) had a 3.0-kb *Hind*III band and the 1.5-kb *Msp*I band seen faintly also in Fig 5, lanes 15 and 18 could not be digested. Analysis of genomic DNA samples from leukocytes of 30 normal controls did not show these unique restriction fragments to be present (not shown). These data suggest rearrangements, deletions, or insertions have occurred within the gene and promoter sequences on at least one allele.

Eukaryotic gene expression may be inhibited by methylation of promoter cytosine bases [49]. Therefore, we compared parallel digestions with methylation-sensitive (*Hpa*II) and -insensitive (*Msp*I) restriction enzymes to detect methylation at 5′-GGCC-3′ sites within the TGase I promoter (Fig 5*A*). If the DNA were unmethylated, *Hpa*II and *Msp*I would be expected to produce identical fragments. Two identical 650- and 500-bp fragments were seen with

Figure 1. Staining for TGase I with B.C1 using a streptavidin-biotin immunoperoxidase DAB method (DAKO) in tumors and in cultured cells in the skin raft equivalent. *A)* Basal cell carcinoma *(solid arrow)* is negative for TGase I but adjacent skin stains normally *(open arrow). B)* Epidermis overlying the BCE is compressed and suprabasal layers stain with TGase I *(arrow). C,D)* Moderately well-differentiated squamous carcinoma shows little staining. Epidermal hyperplasia with precocious expression of TGase I is present in the normal overlying epidermis *(solid arrow).* The pattern of staining is normal in adjacent epidermis *(open arrow). E)* Well-differentiated squamous cell carcinoma with numerous keratinized horn pearls *(bottom, arrows)* staining positively for TGase I. The epidermis is not hyperplastic and shows normal staining pattern. *F)* Normal human foreskin keratinocytes plated on adult human fibroblasts show membrane expression of TGase I in several layers of stratified epidermis, raft skin equivalent. *G,H)* Human A431 carcinoma cells plated on adult fibroblasts. A few cells at the epidermal-dermal border *(H)* and in the upper layers *(G)* stain for TGase I *(arrows). Scale bar,* 50 μ *(A, B, C, D, G)* and 5 μ *(F, H).*

Table I. Expression of Transglutaminase I In SCC Lines

Line	Western Blot	Northern Blot	RT-PCR[a] pg mRNA/ total RNA
Normal cells			
HUK-confluent	++	+++	0.0086
Fibroblasts			
Neonatal	−	−	−
Adult	−	−	
SSC lines			
Vulvar-ME-180	++++	+++	0.011
Tonsil-1-83	−	−	0.00002
Retromolar-14-83	+++	+++	0.006
SRB-1	+/−	++	+
SRB-12	+/−	+	+
KB	−	+/−	+
A431	+/−	+/−	0.00004

[a] RT-PCR was done with an endogenous competitor and 1 : 1 equivalency was calculated by linear regression where Y = (slope) X + intercept. Y is the ratio of band intensities (competitor/endogenous) and X = pg added competitor/250 ng total RNA. The value of X for Y = 1 (equal amounts of competitor and endogenous RNA) is then multiplied by 4 to give pg/μg total RNA.

both enzymes in all DNAs except in *Hpa*II digestions of 1-83 (Fig 5, *lane 2*) and KB (Fig 5, *lane 8*) DNA. This suggests that GGCC sites have been methylated in the promoters of these lines. In addition, the 1-83 line contained a unique *Msp*I band just below the 650-bp band (Fig 5, *lane 13, circled*). From this we conclude that a deletion may have occurred in one allele, affecting the size of the 650-bp fragment in 1-83.

DISCUSSION

SCC tumors and cultured lines vary in their expression of human TGase I protein and mRNA as measured by quantitative Western blotting and RT-PCR. We hypothesize that variation may result from differences in the transcription of TGase I, which might result from mutations or methylation in some cases. Secondly, whereas skin SCC tumors show reduced TGase I, the overlying normal epidermis may express the enzyme abnormally. Tumors are well known to have heterogeneous behavior, response to vitamins or growth factors [24,50], and expression of different antigens, tumor suppressor genes, and oncogenes [51,52]. This is the first demonstration that TGase I genetic mutations and variable mRNA levels are present in human squamous carcinomas.

TGase I participates in squamous differentiation and has been suggested to be a useful marker for tumor progression [24]. Terminal differentiation of keratinizing epidermis requires formation of a cornified envelope catalyzed by epidermal (type 3) or keratinocyte (type 1) transglutaminase [4,13]. TGase I differs from the other transglutaminases by the addition of amino terminal amino acids

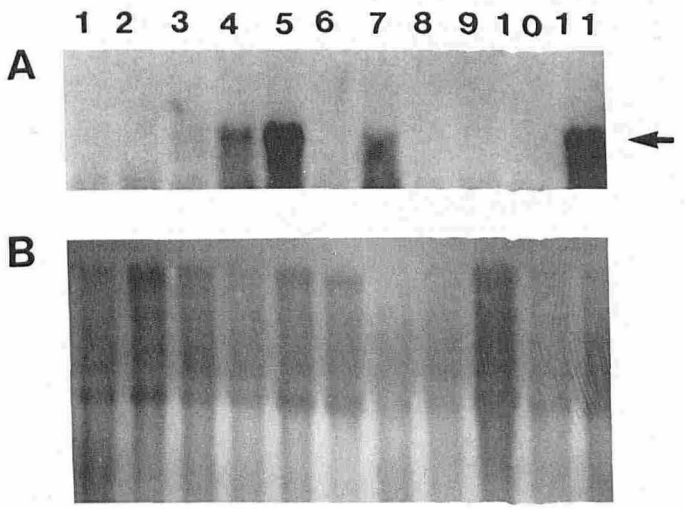

Figure 3. Northern blot analysis of TGase I mRNA levels. Seven micrograms/lane total RNA blotted onto Zetabind membrane were hybridized to ^{32}P-CTP – oligolabeled TGase I cDNA. The filter was exposed for 21 d with intensifying screens [17] *(A)*. For comparison of load, UV shadowing of the membrane was done [17] *(B)*. mRNA ladder (BRL) was used to determine the size of the TGase I mRNA as 2.9 kb *(arrow)*. *Lane 1*, A431; *lane 2*, KB; *lane 3*, SRB-12; *lane 4*, SRB-1; *lane 5*, 14-83; *lane 6*, 1-83; *lane 7*, ME-180. Controls: *Lane 8*, human adult fibroblasts from skin of breast reduction; *lane 9*, human neonatal foreskin fibroblasts; *lane 10*, human foreskin keratinocytes at 70% confluency; and *lane 11*, human foreskin keratinocytes at confluency.

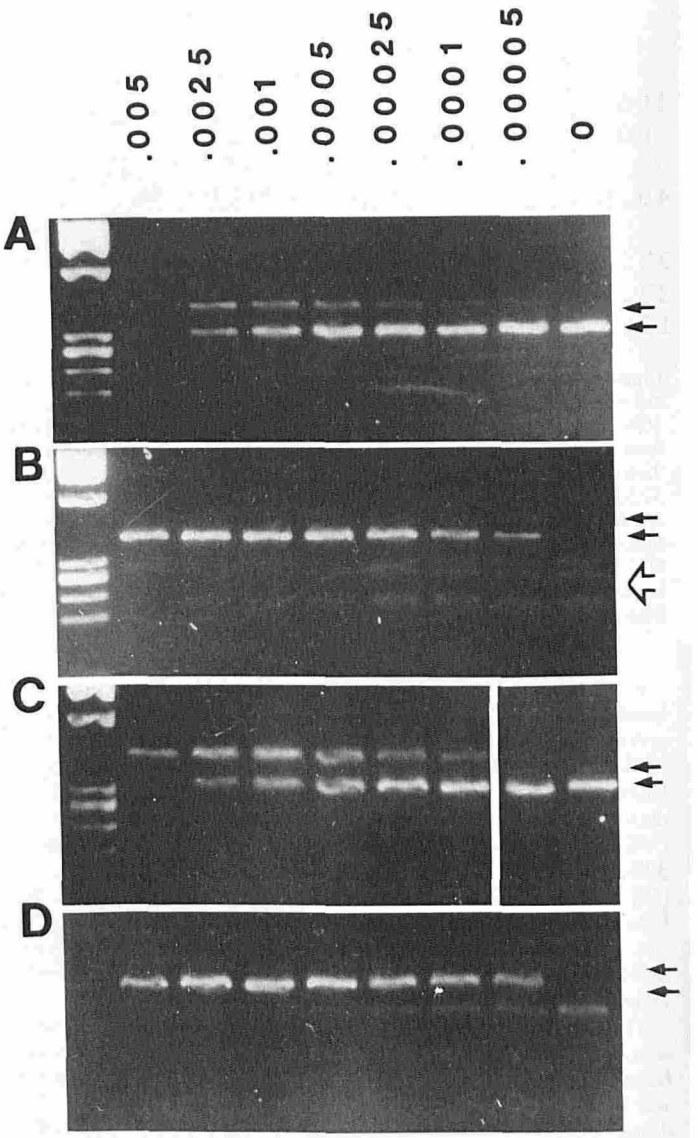

Figure 4. Agarose gels stained with ethidium and visualized under UV light contain quantitative RT-PCR cDNA fragments from competitor riboprobe *(top band, upper arrow)* and from endogenous mRNA *(bottom band, lower arrow)*. Levels of TGase I mRNA derived from cDNA were quantitated in keratinocytes and SCC lines. Following cDNA synthesis, the products were split into eight tubes containing dilutions of competitor (pg amount shown above *lanes*) and were subjected to 35 cycles of PCR. Ten microliters of each reaction was loaded onto 4% Nuseive Agarose Gels and electrophoresed in Tris-EDTA-acetate buffer. Competitor alone is in *lane 2* and no competitor is in the *last lane*, as control. Extraneous bands are shown by *open arrows*. DNA V markers are in the *first lanes* (Boehringer-Mannheim). Cultured human foreskin keratinocytes *(A)*, SCC 1-83 *(B)*, SCC 14-83 *(C)*, and A431 *(D)*.

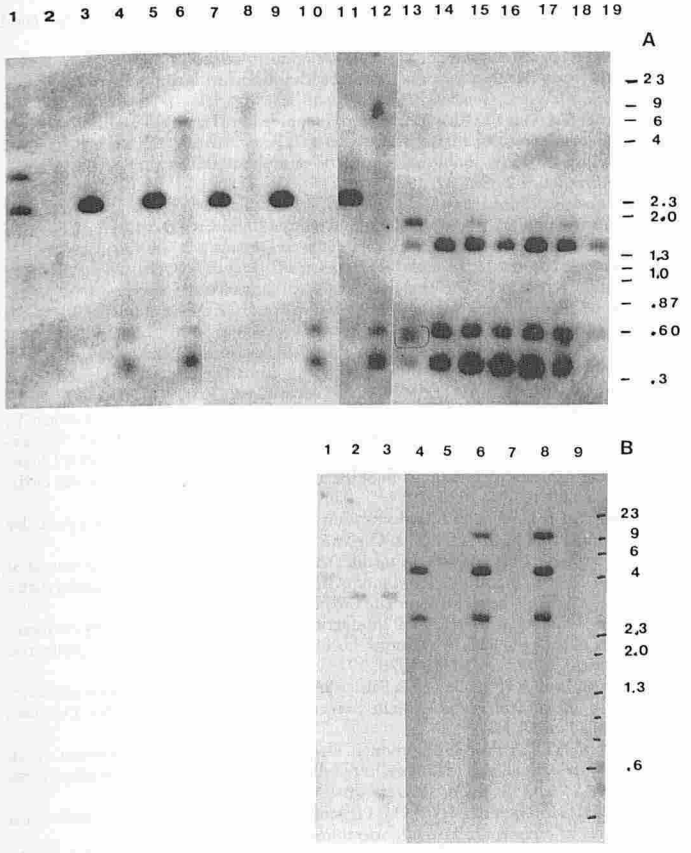

Figure 5. Southern blot of SCC genomic DNA (10 μg/lane) cut with restriction enzymes and hybridized to a genomic promoter 2.3 *Hind*III fragment *(A)* or a 1.5-kb TGase I cDNA beginning at the active site *(B)* described previously [17]. Lambda *Hind*III and Phi X174 fragments (BRL) were used as size markers. Filters were exposed for 5 d to Kodak XAR film with intensifying screens. *A)* Restriction fragments that hybridize to the 2.3-TGase I promoter fragment are shown as follows: *Hind*III digestions *(lanes 1,3,5,7,9,11)*, *Hpa*II digestions *(lanes 2,4,6,8,10,12)*, and *Msp*I digestions *(lanes 13–19)*. DNAs include SCC 1-83 *(lanes 1,2,13)*, SCC 14-83 *(lanes 3,4,14)*, A431 *(lanes 5,6,15)*, KB *(lanes 7,8,16)*, ME-180 *(lanes 9,10,17)*, SRB-1 *(lane 18)*, and foreskin keratinocytes *(lane 19)*. *B)* DNA restriction fragments that hybridize to the TGase I partial cDNA insert [17] were digested with *Msp*I *(lanes 1,2,3)*, *Hind*III *(lanes 4,6,8)*, and *Hpa*II *(lanes 5,7,9)*. DNAs include 1-83 *(lanes 1,4,5)*, 14-83 *(lanes 2,6,7)*, and A431 *(lanes 3,8,9)*.

important for myristylation and membrane anchorage [53,54]. The enzyme is localized in the cytoplasm as well as in the membrane, suggesting functions in addition to cornified envelope formation [20]. TGase I is also homologous to an erythrocyte structural protein (band 4.2 protein) and to other transglutaminases including Factor XIIIa, which stabilizes the fibrin clot [3,17,54]. Thus we speculate that TGase I may play a role in determining keratinocyte cell structure, membrane integrity, and possibly epidermal cohesiveness, which may be important in progression to malignancy and in metastasis.

Some pulmonary carcinomas with squamous character lack TGase I expression uncoupled from another squamous differentiation marker, involucrin [18]. Involucrin has been suggested to distinguish squamous malignancy from dysplasia [47]. In the fresh SCC tumors shown here, only the two most well-differentiated SCCs stained positively for TGase I, whereas the others were negative. Similarly, the well-differentiated SCC lines ME-180 and 14-83 showed the greatest levels of protein expression, equal to or greater than those of cultured foreskin keratinocytes grown in low calcium. Because calcium is known to increase TGase I expression, the level of protein expression of keratinocytes in low calcium may not be directly comparable to the levels in SCCs in high calcium in this particular study. The protein levels were in good correlation with the levels of mRNA detected (except for SRB-1), suggesting that transcriptional control is important for determining the levels of TGase I. Transcriptional regulation has been suggested for TGase I expression in keratinocyte culture in response to calcium, retinoic acid, and phorbol esters [12,21,23,24,55–58]. It is possible that variation in cornification and expression of TGase I in SCC lines or tumors [21] could reflect their degree of responsiveness to retinoids in serum [24,26].

Of interest, both BCCs and SCCs that lack TGase I appear to induce precocious expression of the enzyme in the overlying normal epidermis. This suggests that TGase I expression may be induced in overlying tumors. TGase I immunoreactivity to B.C1 is seen just under the stratum corneum [5,59], although we have reported previously, using *in situ* hybridization [17], that TGase I mRNA is present in all epidermal layers and is markedly increased in psoriatic lesions. Both SCC and BCC tumors have been shown to express TGase I mRNA by *in situ* hybridization as well (Schroeder, Duvic [abstr.] Clin Res 529A, 1991). Others have reported a more restricted localization of the mRNA to the suprabasal layers [59].

Our study differs from other studies of TGase I in squamous cell carcinomas [24,26,28] in that we measure both protein and mRNA in parallel and quantitatively with new highly sensitive methods [26,28,36]. Our data suggest that TGase I protein expression may be controlled by mRNA transcription levels in SCC lines, and is correlated with state of differentiation. Quantitative RT-PCR was a more sensitive method for mRNA detection but showed good correlation with quantitative Northern blots.

With the cloning of the cDNAs and genes for two distinct epidermally derived transglutaminases, TGase I (keratinocyte) [17,54,60–62] and TGase III (epidermal) [61], elucidation of molecular mechanisms for the heterogeneity of transglutaminase expression are possible. Loss of gene expression in tumors can result from the deletion of all or part of one or both alleles or from methylation of key promoter regions controlling transcription. The TGase I gene was detected in all SCC DNAs studied but abnormal genomic fragment sites were noted in two lines that produced very low levels of TGase I mRNA and no detectable TGase protein.

The 1-83 line had abnormal RT-PCR fragments and unique genomic restriction fragments in both the promoter and gene region, suggesting that at least one of the alleles for this gene has structural mutations that affect multiple enzyme sites. Although restriction enzyme-length polymorphisms or partial digestions cannot be excluded, the presence of multiple restriction fragment changes is more likely due to a rearrangement, insertion, or deletion.

Tumor lines in culture for many years may lose chromosomes or accumulate genetic mutations, so the significance of these findings for the development or progression of SCC is not yet known. The SCC 14-83, SRB-1, and SRB-12 lines have been in culture as long as 1-83, yet do not have detectable changes in their TGase I genes. No cytogenetic changes in chromosome 14, which contains the TGase I gene [17,63] have been found in the 1-83 line [29]. Analysis of DNA from fresh tumors would be helpful in determining the importance of these observations.

The methylation pattern of the promoter of the 1-83 and KB lines appears to be altered relative to other SCC and normal cell lines, as studied by methylation-sensitive enzymes. One allele of 1-83 may also contain a deletion that affects a 650-bp *Msp*I fragment. Thus, the 1-83 line may have multiple changes including methylation and structural genomic mutations, resulting in lowered expression and truncated RT-PCR products.

The variation in the expression of TGase I enzyme appears to correlate with the level of squamous differentiation, resulting perhaps from several mechanisms affecting RNA transcription. Two of these may include methylation or mutations in the DNA promoter or gene structure. This is the first evidence to suggest that mutations within the TGase I gene and promoter are associated with human squamous cell carcinoma. The full function of TGase I is as yet

unknown but warrants further investigation with respect to squamous differentiation and development of malignancy.

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