Matthias Ulbrecht[●], Bernd Rehberger[●], Isolde Strobel[▼], Gerald Messer[▼], Peter Kind[▼], Klaus Degitz[▼], Thomas Bieber[▼] and Elisabeth H. Weiss[●]

Institut für Anthropologie und Humangenetik[●] and Dermatologische Klinik und Poliklinik[▼] der Ludwig-Maximilians-Universität, München

HLA-G: expression in human keratinocytes *in vitro* and in human skin *in vivo**

Classical, polymorphic major histocompatibility complex class I molecules are expressed on most nucleated cells. They present peptides at the cell surface and, thus, enable the immune system to scan peptides for their antigenicity. The function of the other, nonclassical class I molecules in man is controversial. HLA-G which has been shown by transfection experiments to be expressed at the cell surface, is only transcribed in placental tissue and in the fetal eye. Therefore, a role of HLA-G in the control of rejection of the allogeneic fetus has been discussed. We found that HLA-G expression is induced in keratinocytes by culture *in vitro*. Three different alternative splicing products of HLA-G can be detected: a full length transcript, an mRNA lacking exon 3 and a transcript devoid of exon 3 and 4. Reverse transcription followed by polymerase chain reaction also revealed the presence of HLA-G mRNA *in vivo* in biopsies of either diseased or healthy skin.

1 Introduction

The human major histocompatibility complex, the HLA complex, contains three so-called classical, polymorphic class I loci coding for ubiquitously expressed cell surface molecules which regulate self/nonself recognition by the immune system, since they bind selected peptides from the cytoplasm and "present" them at the cell surface for inspection by T lymphocytes [1]. Three additional loci (class Ib) coding for invariant HLA class I heavy chains have been characterized [2]. Whether these molecules have a function in immune recognition is controversial. Certain mouse class Ib molecules, however, have been shown to bind and "present" bacterial or heat-shock protein-derived peptides to cytotoxic T lymphocytes [3–5]. Recently, a non-HLA-encoded class I molecule, CD1b, has been demonstrated to restrict the response of human $CD4^-8^-$ $\alpha\beta$ TCR^+ T cells for *Mycobacterium tuberculosis* [6]. In contrast to the classical loci, the transcription of the class Ib genes is often restricted to few tissues, supporting a specialized function of these molecules.

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Due to its developmentally regulated expression, the HLA-G gene [7–9] is unique among the class I gene family. Abundant transcript is only present in cytotrophoblast cells of the placenta [10, 11], although low amounts of HLA-G mRNA were also detected in fetal eye [9], fetal liver [12], thymus [13] and sperm [14]. Moreover, extensive alternative splicing of the HLA-G primary transcript leading to the synthesis of three different α chains has been reported [15]. In contrast to the full length polypeptide, two isoforms lacking either the $\alpha 2$ domain or both the $\alpha 2$ and $\alpha 3$ domains were detected. Such isoforms should be incapable of binding peptides and β_2 microglobulin. Nothing is known about the developmental regulation of the production of alternative forms to date.

2 Materials and methods

2.1 Preparation and cultivation of keratinocytes

Split-thickness skin was washed several times in PBS pH 7.3, then cut into 0.5 × 2 cm pieces and incubated for 90 min at 37 °C in 0.5 % trypsin type XI (Sigma Chemie GmbH, Deisenhofen, FRG). The epidermis was peeled off and vigorously agitated in RPMI 1640 (Biochrom, Berlin, FRG) containing 10 % FCS (Biochrom) and 0.01 % DNase I (Sigma). The resulting cell suspension was filtered through sterile gauze and washed twice in supplemented RPMI 1640. The viability of these epidermal cells was ≥ 90 %, as determined by trypan blue exclusion. Cells were either immediately lysed (freshly isolated keratinocytes; fKC) or subjected to further culture (cultured keratinocytes; cKC). For this purpose, single-cell suspensions were resuspended in supplemented RPMI 1640 and incubated overnight at 37 °C and 5 % CO_2 in culture flasks either coated or not with collagen G (Biochrom). After incubation, nonadherent cells were removed and adherent cells cultured for 14 days until confluence in DMEM (Flow Laboratories) supplemented with 10 % FCS, 25 mM Hepes-buffer (both Biochrom), 1 % antibiotics/antimycotics, 2 mM L-glutamine, 5 mM sodium pyruvate, and 1 % nonessential amino acids (aa) (all Gibco BRL, Eggenstein, FRG).

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Correspondence: Elisabeth H. Weiss, Institut für Anthropologie und Humangenetik der Ludwig-Maximilians Universität, Richard-Wagner-Str. 10/I, D-80333 München, FRG (Fax: +49/0-89-5203-286)

Abbreviations: **3′UT:** 3′untranslated region **RT-PCR:** Reverse transcriptase-PCR **cKC:** Cultured keratinocytes **fKC:** Freshly isolated keratinocytes **aa:** Amino acid

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2.2 Human cell lines and stimulation

The B-LCL LG2 (HLA-type:-A2, -B27, -Cw1) and the choriocarcinoma cell line Jeg-3 were cultured in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, and 1% penicillin/streptomycin (all Gibco BRL). Culturing and stimulation with IFN-γ of the epidermoid carcinoma cell line A-431 or of keratinocytes kept without collagen layer has been described elsewhere [16].

2.3 Northern blot analysis

RNA from human placenta, cell lines, and keratinocytes was prepared according to the procedure of Chirgwin et al. [17]. Ten micrograms of total RNA was separated on formaldehyde agarose gels and transferred to Hybond-N^+ membrane (Amersham Buchler, Braunschweig, FRG) in 0.05 N NaOH for 2 h according to manufacturer's recommendation. The filters were sequentially hybridized with the following probes: the antisense oligomer P3 (5′-CTC CTT TGT TCA GCC ACA TTG GCC-3′, exon 3, aa 149–157) and the antisense oligomer P3′UT [5′-GGG CTG GTC TCT GCA CAA AGA GA-3′, 3′ untranslated region (3′UT)] both specific for HLA-G; the antisense oligomer o-LG2-C1 specific for HLA-E [18]; a 3′UT 550 bp HLA-A-specific probe [18]; a 3′UT 450 bp HLA-B-specific probe [18]; and finally a cytokeratin K5-specific 581 bp probe containing 95 bp of 5′UT and the sequence coding for the first 165 aa amplified from cDNA with the oligomers K5-11a (5′-AGA GCC ACC TTC TGC GTC CTG C-3′) and K5-11b (5′-CTG GAT GCT GGG GTC GAT TTG C-3′) [19]. Oligonucleotides were labeled with terminal deoxynucleotidyl transferase (Boehringer Mannheim, Mannheim, FRG) and [α-^{32}P] dATP (Amersham) [20]. Purification, hybridization, and washing at 65 °C were performed as described [18, 21]. Probes were labeled by the random primer method [22] with [α-^{32}P] dATP (Amersham). Hybridization and washing at 65 °C were done as described [23].

2.4 Reverse transcriptase (RT)-PCR analysis of cultured cells

For first strand cDNA synthesis, 1 μg of total RNA was denatured 3 min at 94 °C in 5 μl of 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol plus 100 ng of either HLA-G-specific oligomer P3′UT or a pan HLA class I antisense oligomer P4 (5′-GCC AGG TCA GTG TGA TCT CCG C-3′, exon 4, aa 211–218 [24]). After an annealing step for 10 min at 55 °C, the reaction volume was raised to 10 μl with the same buffer plus 0.5 mM dNTP, and 200 U of Moloney murine leukemia virus reverse transcriptase (Gibco), and cDNA synthesis was carried out for 60 min at 37 °C. Each cDNA sample was diluted 1/3 and 1 μl was amplified in 20 μl PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3, 1 mM $MgCl_2$), 200 μM dNTP, 1.25 U Taq polymerase (Boehringer) and 12.5 ng of each amplification primer [25]. The following primer combinations and amplification protocols, run on a PCR Thermal Reactor (Hybaid, Teddington, GB) were used: P2–P4: 5 min 94 °C, 40 cycles: 1 min 94 °C, 1 min 62 °C, 2 min 72 °C; 10 min 72 °C; P2 (5′-TTG GGA AGA GGA GAC ACG GAA CAC-3′, exon 2, aa 59–67)-P3 and P2-P6/3′UT (5′-CAG CTG TTT CAC ATT GCA GCC TG-3′, exon 6 3′UT splice junction of HLA-G): 5 min 94 °C; 40 cycles: 1 min 94 °C, 1 min 55 °C, 1 min 72 °C; 10 min 72 °C. PCR products were analyzed either on 1.2% agarose gels containing ethidium bromide or on 5% nondenaturing polyacrylamide gels stained with ethidium bromide after the run.

2.5 RT-PCR analysis of skin biopsies

Skin biopsies, 5 mm in diameter, were lysed in 4 M guanidinium thiocyanate using an ultraturrax device (IKA, Staufen, FRG). Total RNA was isolated by the method of Chomczynski [26]. First strand cDNA synthesis of 1 μg of total RNA primed with oligo-p$(dT)_{15}$ (Boehringer) was performed as described above with the following modification: initial denaturation was carried out at 65 °C and annealing temperature was set to 28 °C.

The primers P2 and P3 were used to amplify HLA-G1 from 2 μl of the diluted cDNA reaction by hot start PCR with AmpliWax PCR Gem 100® (Perkin Elmer, Neuried, FRG) according to manufacturers recommendations but tailoring the reaction mix to half of the volume. The protocol, run on a Prem thermocycler (Lep Scientific, Milton Keynes, GB) was: 94 °C, 5 min; 40 cycles: 94 °C, 1 min; 70 °C, 1 min, 72 °C, 1 min and finally 72 °C, 10 min. Amplification of HLA-E cDNA was performed as described [18]. The PCR products were analyzed on 1.5% agarose gels containing ethidium bromide.

3 Results

3.1 Isolation of HLA-G mRNA transcription in human keratinocytes *in vitro*

During the analysis of various tissues for differential expression of HLA class I loci by Northern blot analysis, an HLA-G-specific antisense oligomer derived from exon 3 (P3 see Fig. 2a) was found to give a strong signal with RNA preparations of normal human keratinocytes (cKC 3) maintained in culture for 14 days on a collagen matrix (Fig. 1).

These cultured keratinocytes contained at least twice as much HLA-G mRNA as the human trophoblast-derived choriocarcinoma cell line Jeg-3 and four times more than placenta. The level of HLA-A, -B, and -E-specific transcripts in these cells was very low, similar to that found in placenta and Jeg-3. No HLA-G transcript could be detected in RNA of fKC 2 of a different donor. The human epidermoid carcinoma cell line, A-431, also did not contain a detectable HLA-G-specific transcript. Whereas keratinocyte cultures grown on collagen matrices expressed large amounts of HLA-G mRNA, only a weak HLA-G induction was observed in subsequent keratinocyte preparations isolated from two other skin specimens (cKC 1 and cKC 2) and maintained in culture without collagen layer for the same time. IFN-γ treatment did not result in a significant increase of HLA-G mRNA in keratinocytes (cKC 1) or in the A-431 cell line, whereas a strong induction of HLA-A, -B and -E mRNA was observed. All keratinocyte preparations have abundant cytokeratin K5 mRNA, characteristic for basal keratinocytes [27]. Only a weak K5 mRNA signal is present in A-431 cells. The specificity of our HLA-G

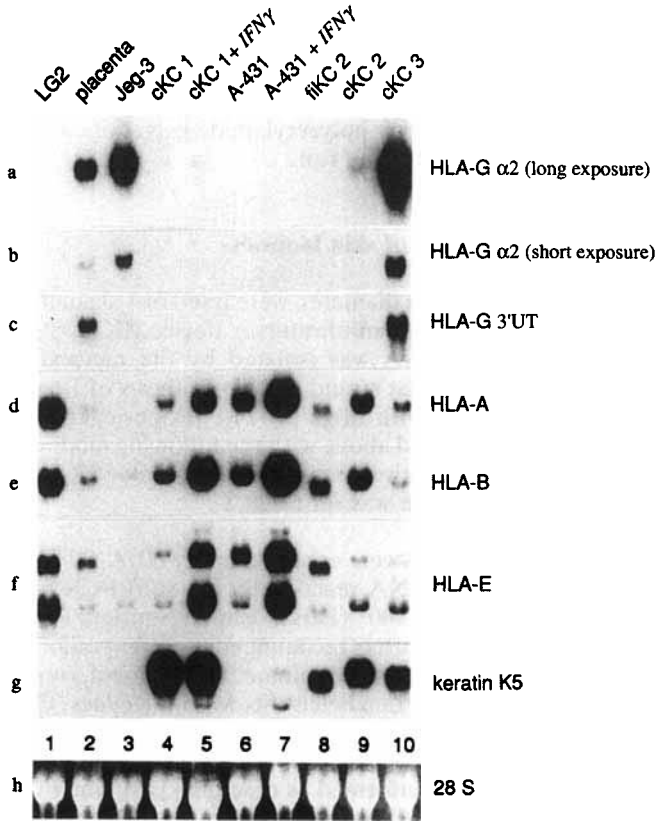

Figure 1. Detection of HLA-G mRNA by Northern blot analysis. Ten micrograms of total RNA per lane were separated on a formaldehyde agarose gel, transferred onto Hybond-N$^+$ and probed with: the HLA-G-specific antisense oligomer P3 (long exposure) (a); P3 (short exposure) (b); the HLA-G-specific antisense oligomer P3'UT (c), a HLA-A-specific probe (d); a HLA-B-specific probe (e); HLA-E specific antisense oligomer o-LG2-C1 (f); a cytokeratin K5-specific probe (g). The amount of RNA loaded is visualized by the ethidium bromide staining of the 28S rRNA (h).

signal was confirmed by RT-PCR analysis of total RNA from cKC 3, using oligonucleotides derived from exon 2 (P2) and exon 3 (P3) (Fig. 2a) of HLA class I-specific cDNA primed with antisense oligomer P4, subcloning the amplified 249-bp fragment and sequence determination of three subclones (data not shown). The HLA-G sequences found are identical to two previously published alleles [7–10]. Detection of HLA-G transcripts by RT-PCR confirmed the results of the Northern blot analyses: cells negative by Northern blot analysis remained negative (Fig. 2b: A-431 and fKC 2).

3.2 Alternative splicing of HLA-G in keratinocytes

Our original HLA-G-specific probe is an oligonucleotide derived from exon 3, detecting the normal full length HLA-G mRNA (HLA-G1) coding for the complete heavy chain (Fig. 2a). The use of an oligomer derived from the 3'untranslated region (P3'UT) of HLA-G, which should hybridize to all three HLA-G transcripts, indicates that a large fraction of the HLA-G mRNA in keratinocytes is present as normally spliced HLA-G1 mRNA. Therefore, the lack of a strong HLA-G mRNA induction in preparations cKC 1 and cKC 2 cannot be explained by preferential alternative splicing of a HLA-G transcript due to allelic differences or culture conditions.

Since Northern blots did not resolve the three HLA-G transcripts, RT-PCR assays were employed to investigate the alternatively spliced HLA-G mRNA species (HLA-G2 lacking exon 3, and HLA-G3 lacking both exon 3 and exon 4). No RT-PCR primer combination resulted in a clean amplification of all three transcripts simultaneously. We, therefore, used different primer sets to detect each mRNA form in either oligo(dT)-primed cDNA (data not shown) or HLA-G-specific cDNA primed with the 3'UT oligonucleotide (P3'UT) (Figs. 2 and 3). We found that

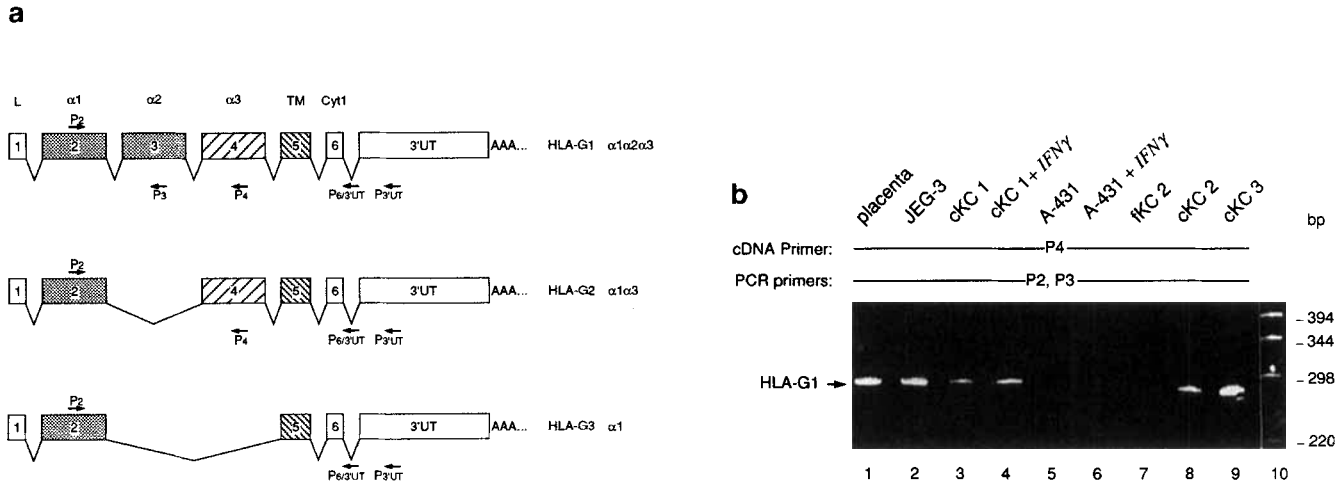

Figure 2. (a) Alternative splicing products of HLA-G. Boxes indicate the coding exons 1–6 and the 3'UT. Localization of the oligonucleotides used in this study are shown with arrow heads indicating the 5' to 3' orientation. Sense primers are depicted above and antisense oligomers below the boxes. P6/3'UT is complementary to the end of exon 6 and the beginning of the 3'UT (exon 6/3'UT splice junction) of the different HLA-G species. The domain structure of the extracellular part of the various translation products is shown on the right. (b) Detection of HLA-G1 by RT-PCR in cultured keratinocytes, placenta and Jeg-3 cells. Primers used for cDNA synthesis and amplification are indicated. The primer combination P2 and P3 amplifies specifically a 294 bp fragment only present in HLA-G1. The PCR products were analyzed on a 5% polyacrylamide gel stained with ethidium bromide.

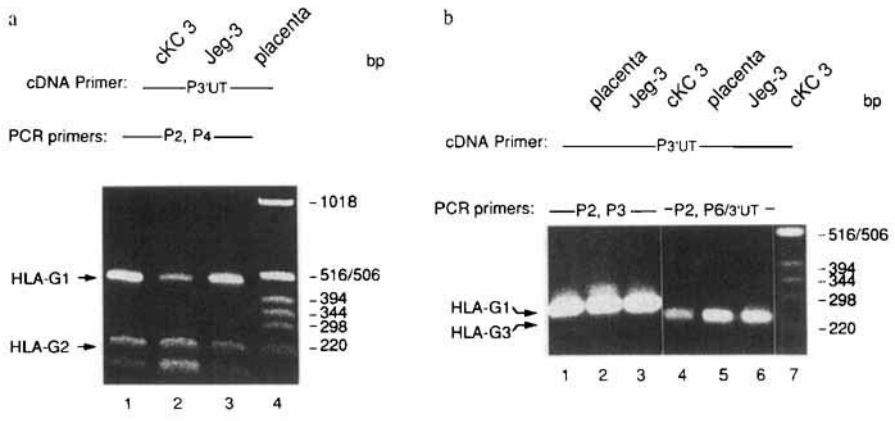

Figure 3. Amplification of the isomers of HLA-G expressed in cultured keratinocytes, placenta and Jeg-3 cells. Primers used for cDNA synthesis and amplification are indicated above each panel. (a) Amplification with primers P1 and P4 results in a 475-bp fragment specific for HLA-G1 and a 200-bp amplificate derived from HLA-G2. (b) Amplification with P2 and P6/3'UT detects only a 250-bp fragment derived from HLA-G3 (right), although the presence of HLA-G1 is shown by amplification with P2 and P3 (left). The PCR products were analyzed on 1.2 % agarose gels containing ethidium bromide.

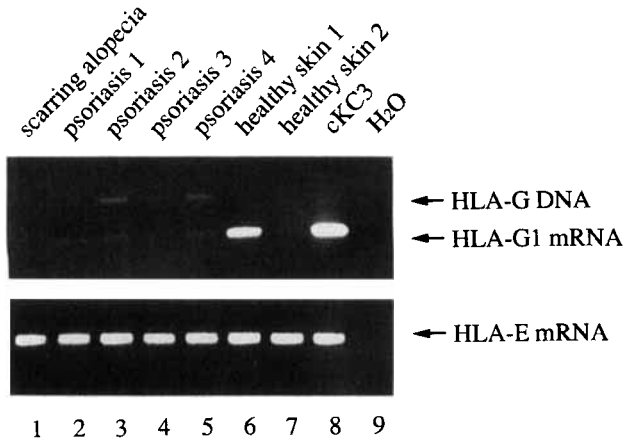

Figure 4. Detection of HLA-G1 (upper panel) and HLA-E (lower panel) by RT-PCR in biopsies of human skin. HLA-G1 from cDNA primed with oligo dT was amplified with the primers P2 and P3 (upper panel). Amplification products derived from cDNA and contaminating genomic DNA are indicated. HLA-E from the same cDNA was amplified as described [18] (lower panel). Biopsies were obtained from different individuals except for scarring alopecia (lane 1) and healthy skin 2 (lane 7). Biopsies from healthy skin were excised from the back (lane 6) and the buttock (lane 7). Oligo (dT)-primed cDNA from cKC3 served as a positive control (lane 8).

keratinocytes express all three HLA-G transcripts. However, our results do not allow quantitation since the amplification products strongly depended on the RT and PCR primers.

3.3 Expression of HLA-G in human skin

Since HLA-G1 is the HLA-G isoform most likely to have an immunological function, we performed RT-PCR analyses with P2 and P3 as amplification primers on biopsies obtained from diseased and healthy skin from different localizations and individuals to assess HLA-G1 expression *in vivo*. Though HLA-G1 expression was detectable in psoriatic lesions, healthy skin of the back (Fig. 4) and healthy foreskin (data not shown), it was absent in material derived from a scarring alopecia and sound skin of the buttock (Fig. 4). Amplification of HLA-E cDNA as a control demonstrates that absence of HLA-G1 amplification in some biopsies (Fig. 4, lanes 1 and 7) was not due to different efficiency of cDNA synthesis.

4 Discussion

Previous results indicated that HLA-G transcription is restricted to few distinct tissues of the fetus [10–12, 28], thymus [13] and sperm [14]. In the placenta, HLA-G and HLA-A,B gene expression seem to be inversely related ([10], Fig. 1). Here, we present evidence of HLA-G expression in non-fetal tissue *in vitro* and *in vivo*. HLA-G mRNA could be induced by *in vitro* culture in all keratinocyte preparations, which also led to an increase in HLA-A and B transcripts, but the levels of HLA-G mRNA varied dramatically (see the weak signal in cKC 1 and cKC 2 versus cKC 3 in Fig. 1). In this experiment keratinocytes expressing high levels of HLA-G (cKC3) were cultured on collagen G. It was, thus, intriguing to test whether the extracellular matrix might deliver an inductive signal for HLA-G expression. This seems not to be a general phenomenon since keratinocytes from another donor only demonstrated a moderate induction of HLA-G mRNA regardless of whether they were grown on collagen G or not (data not shown). Since preliminary data demonstrate that HLA-G transcription can be induced in cultured keratinocytes by a variety of stimuli, differences in culturing conditions other than collagen G might account for the observed differences in induction. The signals and factors regulating HLA-G transcription are not known so far, but our results support previous findings that these are unique and possibly transient in action. The HLA-G enhancer has a deletion in the IFN-responsive element, which could explain its unresponsiveness to IFN treatment (Fig. 1, [7]). Since two HLA-G alleles are expressed in cKC 3 cells, the possibility of a high expressing HLA-G variant to explain the high amount of specific transcript seems rather unlikely.

Since HLA-G-specific mAb are not available, we could not test the cells for cell-surface expression of a HLA-G encoded molecule. In transfectant systems HLA-G, in contrast to HLA-E, is readily transported to the cell surface indicative of a sufficient pool of endogenous peptides capable of binding HLA-G [2]. Therefore, it has to be assumed that HLA-G can be expressed on the cell surface of keratinocytes in a conformation capable to interact with T lymphocytes. The function of HLA-G and its isoforms is not known so far. Previously, the expression of HLA-G was thought to be limited to the cytotrophoblast, and it was speculated that the HLA-G molecule might regulate maternal/fetal immune recognition or specific non-immunological functions of the placenta [11, 28].

We could also demonstrate that HLA-G is transcribed in human skin *in vivo*. HLA-G1 expression as detected by RT-PCR seemed to be considerably lower in psoriatic skin lesions as opposed to one biopsy of normal skin (Fig. 4). Two other biopsies from the same individuum either from healthy or diseased skin did not have any HLA-G mRNA. Testing a larger sample, comprising also biopsies from other skin lesions and foreskin material (data not shown) did not reveal any obvious association between disease and HLA-G1 expression. This might either reflect on the heterogeneity of the cells in a biopsy or their activation status. Clarifying whether and which keratinocytes express HLA-G in the skin, will help to solve this issue. Furthermore, different HLA-G alleles have been described recently [29]. We cannot rule out the possibility that they might differ in their transcriptional activation by different extracellular stimuli. Finally, since the HLA-G transcript can be alternatively spliced, factors regulating splice preferences also determine the level of HLA-G1 expression. In conclusion, the differential detection of HLA-G mRNA in skin biopsies supports the delicate regulation of HLA-G gene expression. Since the function of HLA-G expression in trophoblast cells is not known, a possible involvement of HLA-G in immune responses of the skin remains speculative.

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