High-power femtosecond-terahertz pulse induces a wound response in mouse skin

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Terahertz (THz) technology has emerged for biomedical applications such as scanning, molecular spectroscopy, and medical imaging. Although a thorough assessment to predict potential concerns has to precede before practical utilization of THz source, the biological effect of THz radiation is not yet fully understood with scant related investigations. Here, we applied a femtosecond-terahertz (fs-THz) pulse to mouse skin to evaluate non-thermal effects of THz radiation. Analysis of the genome-wide expression profile in fs-THz-irradiated skin indicated that wound responses were predominantly mediated by transforming growth factor-beta (TGF-β) signaling pathways. We validated NFκB1- and Smad3/4-mediated transcriptional activation in fs-THz-irradiated skin by chromatin immunoprecipitation assay. Repeated fs-THz radiation delayed the closure of mouse skin punch wounds due to up-regulation of TGF-β. These findings suggest that fs-THz radiation initiate a wound-like signal in skin with increased expression of TGF-β and activation of its downstream target genes, which perturbs the wound healing process in vivo.

Terahertz (THz) radiation consists of electromagnetic waves that propagate at frequencies from 0.1 to 10 × 10^12 Hz, the THz range. This radiation is present in our environment as a part of the solar ray spectrum1–2. THz radiation shows both optical particle-like and electric wave-like characteristics, which has opened new fields of research in physics, chemistry, and medicine3–4. THz techniques are now used for the development of clinical imaging5, cancer diagnostics6,7, security systems8 and telecommunication9. As THz wave is widely exposed to human body with a variety of purposes, we need to characterize the biological response against THz radiation in vivo.

The European project THz-BRIDGE investigated the potential damage of electromagnetic radiation in the THz spectral range to biological systems (http://www.frascati.enea.it/THz-BRIDGE/reports/THz-BRIDGE%20Final%20Report.pdf). THz radiation in the range of 1 to 3 THz, at 0.45 J/cm², failed to alter the cellular activity or differentiation of human keratinocytes, compared to un-exposed cells10. Two independent reports have similarly failed to identify genotoxic effects of THz radiation in human peripheral blood lymphocytes11–12. Recent studies using in vitro systems have shown that THz radiation has non-thermally induced impacts on the DNA stability13–16, which would cause chromosomal aberrations in human lymphocytes17 or alterations in gene expression with accelerated differentiation of mouse stem cells18–19. In particular, Titova et al. applied artificial human 3D skin tissue model and exposed samples in vitro to broadband THz with pulse energy up to 1 µJ to detect the signs of DNA damage in THz exposed artificial skin tissue18.

In this study, we undertook an integrated bioinformatic and functional analysis to identify genetic alterations and following reactions in vivo by THz radiation (Fig. 1A). Unsupervised approach using mRNA microarray was applied to screen THz-responsive genes compared to sham exposed samples. Comparative analysis of the expression profiles showed that THz radiation was mostly similar to wound stimulus, not to burning, neutron irradiation or UV exposure. This confirmed the in vitro model with artificial skin tissues15. Further in silico analysis with the differentially expressed genes (DEGs) provided molecular signature responsive to THz irradiation and we found that the wound healing associated signal was predominantly activated via NFκB1 and Smad3/4-mediated TGF-β signaling pathway. To verify such a mechanism, we exposed THz repeatedly on wounds using an in vivo wound model. Interestingly, we found that over-activated TGF-β signaling with the hyper-inflammatory response delayed the healing process of wounds in THz-irradiated mouse skin.
Results

fs-THz radiation does not affect expression of Hsp70 or histology of exposed mouse skin. C57BL/6J mice were exposed to femtosecond (fs)-THz radiation with a pulse duration of approximately 310 fs [full width, at half maximum (FWHM)] and energy of approximately 0.26 nJ/pulse (Fig. S1A and S1B). The frequency spectrum, using Fourier transform, ranged up to 2.5 THz (Fig. S1C), at an average power density of 0.32 mW/cm². The accumulated pulse energy for 1 hour was up to 1.15 mJ/cm². Within the measurement error of the device (± 0.05 °C), there was no change in temperature of the skin of C57BL/6J mice that were exposed to fs-pulsed THz radiation (Fig. S2A). To evaluate for the presence of THz radiation-induced thermal or nonspecific stress, we measured expression of heat shock protein 70 (Hsp70)19,20 at the mRNA (Fig. S2B) and protein levels19,20 (Fig. S2C). Thermal stimulus on NIH-3T3 fibroblasts was check as a positive control with Hsp70 members including Hspa1a, Hspa4l and Hsph1. We did not observe any notable changes in expression of Hsp70 or in the histology of THz-irradiated versus sham-irradiated skin of C57BL/6J mice (Fig. S2D). These results indicate that we could mine further the non-thermally induced biological consequences by THz radiation with the adopted exposure system.

Characterization of the molecular responses to fs-THz radiation. We used microarrays to compare the gene expression profile of
mouse skin 24 hours after exposure to sham or fs-THz radiation. Through a bioinformatic analysis, we identified 149 differentially expressed genes (DEGs) with a mean fold change of signal intensity $\geq 1.5$ (t-test, $P<0.05$). Within this gene list were 82 up-regulated and 67 down-regulated genes (Fig. 1B; Table S1). Gene set enrichment analysis with the Ingenuity Pathway Analysis software identified the top 10 bio-functions with the significance index of Fisher’s exact test in THz-irradiated mouse skin (Fig. 1C and Table S2). The most significant biological response of 24 hours after THz radiation was analyzed as the “healing” process.

Next, we compared expression of the 149 DEGs in fs-THZ-irradiated mouse skin to gene expression, in datasets from Gene Expression Omnibus (GEO), in the skin of mice following exposure to various physicochemical stimuli, including after a skin wound (Fig. 1D and Fig. S3). Normalized datasets were hierarchically clustered with single-linkage in Euclidean distance, and each of Pearson’s correlation coefficient (Pcc) corresponding to the THz dataset was calculated with a two-tailed test of significance ($P<0.05$). The expression of the 149 genes in fs-THz-irradiated skin was mostly similar in wound-induced mouse skin, but not in skin exposed to other stimuli, such as burning, ultraviolet (UV) exposure, or neutron irradiation.

**THz radiation induces expression of genes involved in wound response.** To validate our results from the analysis of gene expression microarrays, we assayed expression of 7 genes from the top two functional categories, wound healing ($Bmp2$, $Cd44$, $Krt6a$, $Lep$, $Serpine1$, $Sprr1b$, and $Thbs1$) (indicated in Fig. 1D and Fig. S4D) and tissue development ($Cc19$, $Hmgbl$, $Myf6$, $Nfatc1$, $Nr4a1$, $Nr4a3$, and $Vdr$) (Fig. 1C and Table S2), by real-time RT-PCR at 1, 12, 24, and 36 hours after fs-THz radiation in two mouse strains, C57BL/6J (Fig. 2A), BALB/c nude (Fig. 2B and Fig. S4). The expression of selected wound response genes were also changed in punch wound skin of C57BL/6J mice (Fig. 2C). The expression pattern was largely consistent with microarray data (Fig. S4D) and expression of most of the wound response genes was increased by 1 hour after exposure to THz radiation. Increased expression of $Bmp2$, $Cd44$, and $Thbs1$ proteins after fs-THz radiation was verified by immunohistochemical staining (Fig. 2D). Our results establish that the expression pattern induced by THz radiation is analogous to that by wound stimulus.

**Activation of TGF-β signaling in fs-THz-exposed mouse skin.** Since the transcription of wound response genes is regulated in part by cytokines such as TGF-β$^{21–23}$, we verified activation of TGF-β signaling in THz radiation-induced wound responses. First, we detected the enrichment of TGF-β signaling pathway in 149 DEGs by IPA analysis (Fig. 3A). Although TGF-β signaling was slightly increased by gene expression microarray, expression of $Tgfb1$ mRNA increased at 1 hour after THz radiation, by real-time

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**Figure 2** | Molecular characteristics of responses to fs-THz radiation in mouse skin. (A and B) Relative expression of 7 genes ($Bmp2$, $Cd44$, $Krt6a$, $Lep$, $Serpine1$, $Sprr1b$, and $Thbs1$) by real-time RT-PCR, selected from the top enriched bio-function, “healing” (wound response genes) (See Fig. 1C and Table S2), in C57BL/6J (A) and BALB/c nude mice (B). Skin of mice was exposed either to sham or THz radiation. (n = 8 in each group). (C) Expression of wound response genes in an in vivo wound model, 24 hours (h) after THz radiation (each, n = 4). (D) Immunohistochemical staining for $Bmp2$, $Cd44$, and $Thbs1$. The original magnification used for all images was $\times 100$. A magnified region of staining is shown as an inset in the lower right. (A–C, Mean ± standard deviation (SD). *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$).
RT-PCR, and decreased thereafter (Fig. 3B). This result was confirmed in BALB/c nude mice and in an in vivo wound model (Fig. 3B). As a positive control, we treated NIH-3T3 mouse fibroblasts with activators of TGF-β signaling, Activin or TGF-β. Similar to our results in THz-irradiated mouse skin, treatment with Activin or TGF-β increased expression of Tgfb1 and wound response genes (Fig. 3C).

To confirm activation of TGF-β signaling in THz radiation-exposed skin, we investigated the activity of TGF-β signaling-related transcription factors (TFs). Based on the expression of DEGs in our dataset, we identified TFs that could potentially target wound response genes using Ingenuity Knowledge Base software (Fig. 3D). Considering most highly activated state (z-score) and its significance (P value), we selected Smad3 and NFκB1, that are main downstream mediators of TGF-β signaling24,25, to examine wound-induced transcriptional activity in fs-THz-irradiated skin. Next, promoter-wide analysis for the transcription factor binding sites (TFBSs) of the selected TFs targeting wound response genes (Smad3 for Bmp2, Serpine1 and Thbs1; NFκB1 for Bmp2, Cdh44 and Serpine1) was carried out using TRANSFAC® professional (Fig. 3E). From in silico prediction analyses, we conducted chromatin immunoprecipitation (ChIP) assays with primer sets designed to distinguish the promoter of each of the genes listed above (Table S4). In mouse skin exposed to THz radiation, Smad3 bound to the promoters of Bmp2, Serpine1, and Thbs1; NFκB1 bound to the promoters of Bmp2, Cdh44, and Serpine1 (Fig. 3E). Smad3 and NFκB1 also bound to the promoter of target genes in the skin of THz radiation-exposed BALB/c nude mice, wound-induced C57BL/6J mice, and in TGF-β-treated NIH-3T3 mouse fibroblasts (Fig. 3E). Although there was not a large fraction of promoters bound by the Smad3 and NFκB1 TFs, DNA binding was quite specific in mouse skin exposed to THz radiation.

Effect of fs-THz radiation on wound healing. Although we were able to identify activation of TGF-β signaling in fs-THz-irradiated mouse skin, the irradiated area did not show any histologic evidence of wound damage (Fig. S2D). We reasoned that if TGF-β signaling was induced in THz radiation-exposed skin without apparent tissue damage, it may be affecting wound healing. To this end, we made a
4-mm punch wound in the skin of C57BL/6J mice and exposed this wounded skin to daily fs-THz radiation. After 10 days, punch wounds were completely closed in sham-exposed skin. In contrast, fs-THz radiation significantly delayed the time for wound closure (Fig. 4A and 4B). The difference in open wound area was also evident by analysis of tissue histology (Fig. 4A). We observed slower re-epithelialization with bigger scab formation in mouse skin that was continuously exposed to THz radiation. The results of a multiplex enzyme-linked immunosorbent assay (ELISA) for expression of TGF-β family members showed significant elevation of Tgb1 protein both at 5 and 9 days following initiation of THz radiation to wound-exposed mouse skin (Fig. 4C). Thus, elevation of Tgb1 expression may be one explanation for the inhibited wound healing process in THz-irradiated mouse skin. These results are consistent with previous findings that elevated plasma levels of TGF-β inhibit re-epithelialization with scarring26,27 (Fig. 4D).

Discussion

The wide variety of applications for THz technology has raised issues of biological safety due to exposure to THz-associated radiation1. In this study, mRNA expression profiling detected wound responses induced by fs-THz radiation in mouse skin. Although we could not find either macroscopic or microscopic evidence of tissue damage in irradiated skin, our results suggest that THz radiation induces wound-like stimuli in keratinocytes through activation of TGF-β signaling.

Wound response is a dynamic process that includes inflammation, granulation, re-epithelialization, matrix formation, and tissue remodeling28. Keratinocytes have a role in re-epithelialization as a consequence of proliferation and differentiation in basal layers of the epidermis. Although the process of early inflammation is mediated by fibroblasts and macrophages, keratinocytes up-regulate expression of cytokines to recruit neutrophils to sites of injury29,30. Among the up-regulated cytokines, we discovered significant up-regulation of TGF-β signaling 24 hours after mouse skin was exposed to THz radiation. TGF-β signaling has pleiotropic effects on inflammation29. Others have shown that elevated levels of TGF-β in keratinocytes significantly inhibit wound re-epithelialization in transgenic mice overexpressing TGF-β. We have similarly shown that repetitive exposure to THz radiation results in elevated levels of TGF-β and delayed closure of punch wounds in mouse skin.

TGF-β signaling is primarily propagated by Smads and a complex of NFκB29,31. We have shown that THz-sensitive genes are mostly associated with wound response and tissue development. We have further shown that transcriptional activation of these genes is mediated by the transcription factors Smad3/4 and NFκB1. In Fig. 4D we propose a mechanism by which THz radiation promotes a wound response in skin. However, the initial physical interactions between THz radiation and skin that trigger this signaling cascade remain unclear. By abrogating TGF-β-induced stimulation of collagen gene expression, myofibroblast transdifferentiation, and Smad-dependent activity in normal fibroblasts, PPAR-γ may play a physiologic role in regulating the pro-fibrotic response31. We have shown increased levels of cytokines, such as TGF-β, following exposure of skin to THz radiation.

We found that a number of genes related to wound response, such as Bmp2, Cd44, Thbs1, and Serpine1, were specifically altered by THz radiation. UV radiation has also been reported to increase expression of TSP-1 at the mRNA and protein levels, in human skin dermis and dermal fibroblasts27. The transmembrane glycoprotein CD44 and its receptor integrin αvβ3 have been shown to mediate inflammatory responses in a wound-like environment32. The transmembrane CD44 on fibroblasts is activated by TGF-β33,34. In this study, we hypothesize that CD44 expression on fibroblasts is activated by THz radiation, and that this activation is associated with elevated TGF-β signaling and subsequent wound response.

Figure 4 | Delayed wound healing associated with fs-THz radiation. (A) Gross and microscopic time course photographs of skin wounds following treatment with sham or THz radiation. Upper panels: photographs are size-compensated and shown as binary images. Bottom panels: dotted lines indicate the margins between re-epithelialized skin and the original wound site. An arrow denotes strongly stained parts of scab spread in wounds. Histologic images were originally magnified at ×40 (scale bar: 500 μm). (B) Quantitative analysis of the open wound area in sham- or THz-irradiated skin (n = 10 in each group). (C) ELISA analysis for Tgb1 in wounded skin following treatment with sham or THz radiation (each, n = 3). (D) Proposed model of wound response induced by fs-THz radiation. TGF-β signaling is activated in THz-exposed skin, which triggers transcription of wound healing-responsive genes via activation of the TFs, Smad3/4 and NFκB. (B and C, Mean ± SD. * P<0.05; ** P<0.01; *** P<0.001).
is currently thought to be the main cell surface receptor for the glycosaminoglycan hyaluronate. Although UV radiation reduced expression of CD44 and hyaluronate in the epidermis of hairless mice, topical retinol or retinoic acid increased the basal level of epidermal CD44. The responses to a pulse of THz radiation may overlap with responses to a series of environmental stresses such as UV, LED, and retinol.

There have been a few reports suggesting direct interactions between THz radiation and biomolecules, which regulate the effects of THz radiation. The structure and function of biomolecules are dynamically controlled via their interactions with water. Based on the fundamental principle that the stretching and bending of hydrogen bonds is affected in the THz domain, it is conceivable that the vibrational, torsional, and librational dynamics of molecules near water are affected by exposure to THz radiation. Alexandrov et al. also proposed that THz radiation may significantly perturb the natural state of dsDNA. Thus, THz radiation may alter important molecular processes involved in gene expression and DNA replication. Others have demonstrated augmented expression of adipogetic differentiation genes (Adiponectin, Glut4, Fabp4, and Pparγ) in mouse stem cells after 9 hours of THz exposure. In our experimental conditions, within 1 hour of exposure of mouse skin in vivo to THz radiation, prediction analysis of putative transcription factors governing DEGs showed slight activation of PPAR-γ (Table S3). Unfortunately, although we noted a transient change in the expression of Adiponectin, Glut4, Fabp4, and Pparγ 12 hours after THz radiation (data not shown), these four genes were not detectable after filtering for DEGs at 24 hours after THz radiation.

In summary, we demonstrated for the first time that THz radiation produces cellular responses against wound-like stimulation. Although THz radiation barely penetrated the outermost region of the epidermis, absorption of THz radiation increases with frequencies in the THz radiation (data not shown), these four genes were not detectable after 1 hour exposure to THz radiation. After a 1 hour exposure to THz radiation, a full thickness of exposed skin was removed, at indicated times, for analysis. Of the wounds were made on the shaved dorsal skin with a 4-mm sterile biopsy punch (Kai Medical, Honolulu, HI). For the experiments in which THz pulses were delivered to wounded mice, we spread deep delams delivered layer by layer onto the shaved dorsal skin of mice was used to administer THz radiation. In a further experiment to THz radiation, a full thickness of exposed skin was removed, at indicated times, for analysis.
Chromatin-immunoprecipitation (ChIP). Transcriptionally-regulated DNA sites for target genes of NFκB and Smad3 were analyzed with TRANSFAC professional (BIOSAB, Wolfenbüttel, Germany), following standard protocols.41. Promoter sequences for each target gene were identified with Regulatory Sequence Analysis Tools (RSAT; http://rsat.ubh.ac.be), with an Ensemble ID. Thousands of promoter sequences were retrieved with the TRANSFAC database. Potential TF binding sites in the promoter of target genes were scanned. Two candidate sites were selected using the highest matrix similarity and core similarity scores.

Skin tissue sections and cells were fixed in 1% formaldehyde at room temperature. Genomic DNA was extracted from tissue or cells using a Pierce® Agarose Chip kit (Thermo Scientific, Waltham, MA). Instead of sonication, we applied microfluidic nucleolysis, where 1 mg of DNA into a uniform size. Chip grade anti-NFκB1 (Abcam, Cambridge, UK) and anti-Smad3 (Abcam) antibodies were used for immunoprecipitation (IP) with the digested chromatin. Finally, purified DNA samples, including input and IP, were prepared for qPCR detection with the specific primers listed in Table S4. IP with negative control IgG or positive control RNA polymerase II antibody and all the designed promoter primers, as well as primers for Gp96, were validated by PCR amplification and agarose gel electrophoresis. Real-time PCR was carried out with a Mx3005P QPCR System using SYBR® Premix Ex Taq™, according to the manufacturer’s instructions. PCR reactions were performed in triplicate, normalized to the ΔCt value of IP to that of input, and expressed as the percentage of input, with error bars representing standard deviation. A two-tailed Student’s t-test was used to determine statistical significance; significance was accepted at P<0.05.

Measurement of TGF-β. The protein level of TGF-β in mouse skin was measured using a Bio-Plex Pro™ Assays kit, according to the manufacturer’s protocols (Bio-Rad, Hercules, CA). Total RNA from mouse skin was purified to a uniform concentration before measurement in a Bio-Plex® 200 System (Bio-Rad). Beads for anti-TGF-β were mixed with lyses, and the fluorescent intensity was converted into a concentration of TGF-β. Each value was an average of triplicate experiments with standard error. The difference of TGF-β concentration between sham- and THz radiation-exposed skin was considered to be significant for P<0.05.

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