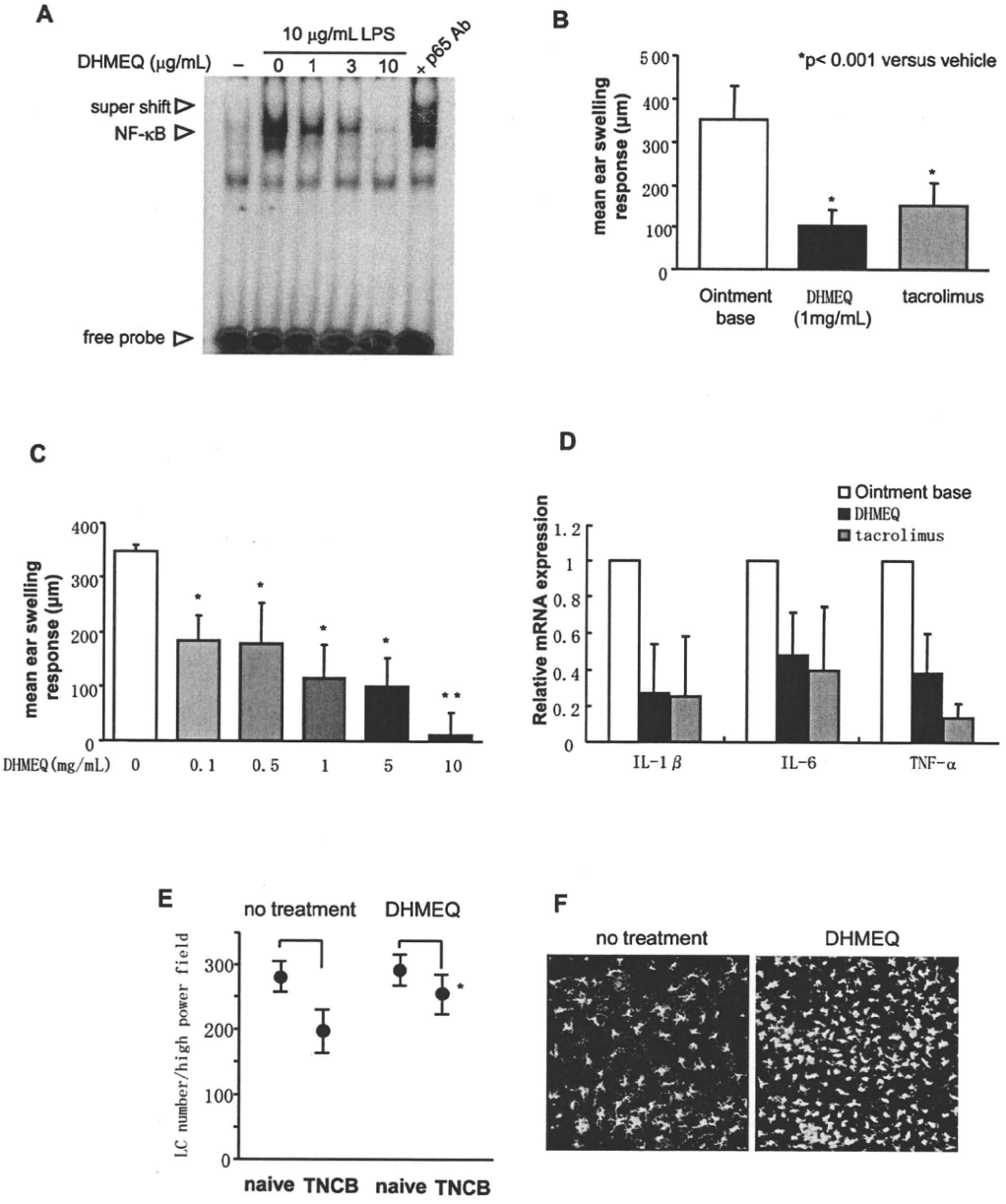

FIG 1. Effect of DHMEQ on contact hypersensitivity response. **A,** DHMEQ effectively inhibited the NF-κB activity in macrophagelike cell line RAW264.7 with LPS stimulation (10 μg/mL). **B,** DHMEQ, tacrolimus, or ointment base was applied topically. All ear-swelling values are shown as means ± SEs (n = 5). $*P < .01$; $**P < .005$. **C,** DHMEQ was applied in various concentrations. **D,** The density of mRNA expression of skin was analyzed by RT-PCR. **E,** The number of LCs was counted. $*P < .05$ (n = 4 for each group). **F,** LC morphology in epidermal sheet preparations derived from untreated *(left)* and DHMEQ-treated *(right)* mice.

In conclusion, we clearly demonstrated that DHMEQ inhibits the contact hypersensitivity response via suppression of inflammatory cytokines and decrease in LC migration. Furthermore, DHMEQ was found to improve AD manifestation of model mice with an efficacy equivalent to that of tacrolimus or betamethasone. DHMEQ may offer a novel therapeutic approach for the treatment of AD.

Asuka Hamasaka, MD, PhD[a]*
Naoya Yoshioka, MS[a]*
Rüichiro Abe, MD, PhD[a]
Satoshi Kishino, PhD[d]
Kazuo Umezawa, MD, PhD[e]
Michitaka Ozaki, MD, PhD[b]
Satoru Todo, MD, PhD[c]
Hiroshi Shimizu, MD, PhD[a]

From [a]the Department of Dermatology, [b]the Department of Molecular Surgery, and [c]the First Department of Surgery, Hokkaido University Graduate School of Medicine, Sapporo; [d]the Department of Medication Use Analysis and Clinical Research, Meiji Pharmaceutical University, Tokyo; and [e]the Department of Applied Chemistry, Keio

University, Yokohama, Japan. E-mail: aberi@med.hokudai.ac.jp, shimizu@med.hokudai.ac.jp.

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*These authors contributed equally to this work.

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A novel non–IgE-mediated pathway of mite-induced inflammation

To the Editor:

In a recent article published in the *Journal of Immunology*, Barrett et al[1] demonstrated that mite and *Aspergillus fumigatus* extracts stimulate the production of cysteinyl leukotrienes from bone marrow–derived dendritic and pulmonary $CD11c^+$ cells through a glycan C–type lectin receptor (Dectin-2) interaction involving FcRγ and Syk signaling that activates arachidonic acid metabolism.

Previously, a number of clinical and experimental observations had called our attention to the existence of important connections between IgE-mediated diseases and leukotriene-mediated inflammation. We had observed that a large proportion of patients with hypersensitivity to nonsteroidal anti-inflammatory drugs (NSAIDs), a condition that is accompanied by increased production of leukotrienes, were atopic and had IgE-mediated sensitivity to mite allergens.[2] This observation has been recently confirmed in another study in which we found significantly increased total and mite-specific IgE levels in patients with NSAID hypersensitivity compared with those seen in healthy control subjects.[3] In tropical countries in which high relative humidity and temperature are optimal for mite proliferation, mite allergens are the main source of sensitization and allergic respiratory disease.

A second clinical observation, also reported by Spanish investigators and others, was that most patients with anaphylaxis after the ingestion of mite-contaminated foods frequently exhibit hypersensitivity to NSAIDs.[4] We designated the association of allergic rhinitis, aspirin/NSAID hypersensitivity, and severe reactions to mite-contaminated foodstuffs as a "new aspirin triad." To understand this association, we performed a study in collaboration with Canadian investigators in which it was demonstrated that mite allergenic extracts inhibited COX-1 *in vitro*. We postulated that mite-induced human allergic diseases could be accompanied, at least in a subset of the patient population, by a dysregulation of leukotriene biosynthesis, metabolism, or both similar to the disturbances described in patients with NSAID hypersensitivity.

In concordance with that hypothesis, various genetic polymorphisms that involve leukotriene C_4 (LTC_4) synthase and cysteinyl leukotriene receptors have been observed in patients with hypersensitivity to NSAIDs; in that context it is important to mention that so-called atopic genes are located in the 5q22-q35 region of human chromosome 5, close to the *LTC4S* gene.

Diverse lines of evidence also support a dysregulation of leukotriene pathways in subjects with mite allergy. Acevedo et al[5] described an association of the A-444C allele of the *LTC4S* gene and IgE response to mite allergens, and we have observed that NSAID-sensitive patients show stronger skin test responses and increased specific IgE antibodies to *Blomia tropicalis* than atopic non–NSAID-sensitive subjects.[3]

Cysteinyl leukotrienes modulate the allergic response, as evidenced in various studies in which it has been shown that leukotrienes enhance IgE and IgG production by human B cells, whereas *LTC4S* knockout mice have a markedly reduced antigen-induced T_H2 pulmonary inflammation. Additionally, it has been demonstrated that IL-4 and IL-13 modulate the number of cysteinyl leukotriene type 1 and 2 receptors on T, B, and antigen-presenting cells.

Furthermore, various groups of investigators have observed that aspirin enhances food-dependent exercise-induced anaphylaxis[6] and facilitates anaphylaxis induced by food allergens.[7-10] These effects could be due to an increased gut permeability, resulting in enhanced opportunity for sensitization at the immunocompetent cell–rich gastrointestinal submucosa.

Bachert et al[11] have proposed a role for staphylococcal enterotoxins, which, through the Vβ receptor on T lymphocytes, allow polyclonal IgE production, including IgE to house dust mite, in nasal polyps, the lungs, and possibly the skin. It would be interesting to investigate in the future whether aspirin-hypersensitive patients with urticaria and angioedema and those with oral anaphylaxis to mites have superantigen-induced immune stimulation

coexistent proliferative epidermal lesions, such as condyloma acuminatum, Bowen's disease, and squamous cell carcinoma, but not in adjacent EMPD areas [3,9,10]. Therefore, HPV infection in these cases is more likely coincidental than causal in the pathogenesis of EMPD, although the precise relationship still needs to be elucidated.

Our findings provide further evidence that HPV infection is unlikely to contribute to the carcinogenesis of EMPD. However, further investigation is required to determine whether or not there is an association between EMPD and other types of HPV that were not detected by the methods used in this study.

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Kazutoshi Murao*
Yoshiaki Kubo
Tsuyoshi Ishigami
Seiji Arase
Department of Dermatology, Institute of Health Biosciences, The University of Tokushima Graduate School, 15-18-3 Kuramoto-cho, Tokushima, Japan

*Corresponding author. Tel.: +81 866 33 7154; fax: +81 886 32 0434
E-mail address: kmurao@clin.med.tokushima-u.ac.jp (K. Murao)

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Letter to the Editor

Chromosome 11q13.5 variant: No association with atopic eczema in the Japanese population

Dear Sir,

A single nucleotide polymorphism (SNP) on chromosome 11q13.5 [rs7927894] has been attracting great attention since Esparza-Gordillo et al. [1] reported highly significant association of a common variant of rs7927894 with atopic eczema (AE) in the German population. In the report, approximately 13% of individuals are homozygous for the SNP and their risk of developing AE is 1.47 times that of noncarriers. Very recently, O'Regan et al. [2] further published interesting results on the association between rs7927894 and AE in a collection of Irish children with moderate-to-severe AE and Irish controls. The association between rs7927894 and AE was replicated in the Irish population ($p = 0.0025$, Chi-square test; odds ratio (OR) = 1.27, 95% confidence interval (CI) 1.09–1.49). Additional analyses performed to test the statistical significance of the rs7927894 SNP having controlled for the presence/absence of the strongly significant *FLG* null genotype indicated that rs7927894 still shows a statistically significant effect ($p = 0.0025$) with an OR of 1.22 (95% CI 1.02–1.26) [2]. Tests for interaction between each of the *FLG* and rs7927894 risk alleles showed no evidence of statistically significant epistatic effects [2]. The rs7927894 association was independent of the well-established *FLG* risk alleles and may be multiplicative in its effects.

In order to clarify whether this common variant is associated with AE also in the Japanese population or not, we evaluated the association between rs7927894 and AE in an cohort of 194 Japanese AE patients we had collected to date and 113 unrelated Japanese control individuals. All the AE patients had been diagnosed with AE based on widely recognized diagnostic criteria [3] or their parents reported a dermatologist's diagnosis of AE (at least once). Majority of AE patients and control individuals were identical to those in a previous study [4]. Using genomic DNA, AE patients and control individuals were screened for the variant allele of rs7927894 on chromosome 11q13, by direct DNA sequencing. In addition, the AE patients and the control individuals were screened for eight *FLG* mutations previously identified in the Japanese population, by restriction enzyme digestion, fluorescent PCR and/or direct DNA sequencing as described previously [4,5].

Case–control association analyses were performed for the variant using Fisher's exact test. In addition, we performed case–control statistical analysis for the common variant allele of rs7927894 after stratification for *FLG* mutations. The rs7927894 on chromosome 11q13 genotype data in the Japanese AE case series and ethnically matched population control series are summarized in Table 1. All alleles were observed to be in normal Hardy–Weinberg equilibrium.

Here we demonstrate that 22.7% and 1.5% of the patients in our Japanese AE case series are heterozygous and homozygous for rs7927894[T], respectively (combined rs7927894[T] allele frequency = 0.129, $n = 388$) (Table 1). rs7927894[T] is also carried by 23.0% of the Japanese control individuals (combined minor allele frequency = 0.115, $n = 226$). There is no statistically significant association between the rs7927894[T] and AE.

After stratification for *FLG* mutations previously identified in the Japanese population, 26.0% and 4.0% of our Japanese AE case series with *FLG* mutations are heterozygous and homozygous for rs7927894[T] (combined rs7927894[T] allele frequency = 0.17, $n = 100$). 21.5% and 0.7% of the Japanese AE patients without *FLG* mutations are heterozygous and homozygous for rs7927894[T] (combined rs7927894[T] allele frequency = 0.11, $n = 288$). There is no statistically significant association between the rs7927894[T] and AE without *FLG* mutations or rs7927894[T] and AE with *FLG* mutations (Fisher's exact test $p = 0.338$). Furthermore, interaction

Table 1
Results of the 11q13.5 SNP and the prevalent *FLG* mutations in 194 Japanese eczema cases and 113 individuals from Japanese control population.

	Eczema cases			Control		
	Total	*FLG* (+)	*FLG* (−)	Total	*FLG* (+)	*FLG* (−)
C/C	147 (75.8%)	35 (70.0%)	112 (77.8%)	87 (77.0%)	2 (50.0%)	85 (78.0%)
C/T	44 (22.7%)	13 (26.0%)	31 (21.5%)	26 (23.0%)	2 (50.0%)	24 (22.0%)
T/T	3 (1.5%)	2 (4.0%)	1 (0.7%)	0 (0%)	0 (0%)	0 (0%)
Total	194	50	144	113	4	109

FLG (+), with *FLG* mutation(s); *FLG* (−), without any *FLG* mutation. Combined rs7927894[T] allele frequency, 0.129 (AE patients, $n = 388$); 0.115 (control individuals, $n = 226$); 0.17 (AE patients with *FLG* mutation(s), $n = 100$); 0.115 (AE patients without *FLG* mutation(s), $n = 288$).

Table 2
Cross-classification of genotypes of rs7927894 and *FLG* used for the interaction analysis.

Genotype		Cases			Controls		
		rs7927894			rs7927894		
		AA	AA	Aa	AA	Aa	aa
R501X	AA	147	44	3	87	26	0
	Aa	0	0	0	0	0	0
	aa	0	0	0	0	0	0
3321delA	AA	141	42	2	87	25	0
	Aa	6	2	1	0	1	0
	aa	0	0	0	0	0	0
S1695X	AA	147	44	3	86	26	0
	Aa	0	0	0	1	0	0
	aa	0	0	0	0	0	0
Q1701X	AA	144	44	3	87	26	0
	Aa	3	0	0	0	0	0
	aa	0	0	0	0	0	0
S2554X	AA	141	41	3	87	26	0
	Aa	6	3	0	0	0	0
	aa	0	0	0	0	0	0
S2889X	AA	133	36	2	86	25	0
	Aa	14	8	1	1	1	0
	Aa	0	0	0	0	0	0
S3296X	AA	141	43	3	87	26	0
	Aa	6	1	0	0	0	0
	Aa	0	0	0	0	0	0
K4022X	AA	144	43	3	87	26	0
	Aa	3	1	0	0	0	0
	Aa	0	0	0	0	0	0
Combined *FLG* null genotype	AA	109	31	1	85	24	0
	Aa	35	13	2	2	2	0
	Aa	0	0	0	0	0	0

AA, wild-type homozygous individuals for each genetic variant; Aa, wild-type/mutant heterozygous individuals; aa, individuals who are homozygous for each of the genetic variants tested.

between each of the *FLG* and rs7927894 risk alleles based on the cross-classification of genotypes in Table 2 showed no apparent epistatic effects.

rs7927894 is located in an intergenic region 38 kb downstream of *C11orf30* (chromosome 11 open reading frame 30) and 68 kb upstream of *LRRC322* (leucine rich repeat containing 32). Both *C11orf30* and *LRRC322* are ubiquitously expressed including skin and peripheral blood lymphocytes [1]. By genome-wide association study for global mRNA expression in lymphoblastoid cell lines from asthmatic children, there was no evidence for a *cis*-regulatory effect of rs7927894 [6]. Thus, regulatory role of rs7927894 on *C11orf30* and *LRRC322* gene expression is questionable. However, we cannot exclude the possibility of a pathogenetic link of rs7927894 to atopic eczema via *C11orf30* and *LRRC322* gene expression in the skin.

Our case–control study in the Japanese population did not confirm the result of Esparza-Gordillo et al. [1] or O'Regan et al. [2] that rs7927894 is at increased risk for AE. The association of rs7927894 with AE was reported in the European population, i.e. in the German population by Esparza-Gordillo et al. [1] and in the Irish population by O'Regan et al. [2]. The present data suggest that the association of rs7927894 with AE established in the European populations is not in the Asian populations, at least in the Japanese population.

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Yukiko Nomura
Department of Dermatology, Hokkaido University Graduate School of Medicine, North 15 West 7, Sapporo, Hokkaido 060-8638, Japan

Masashi Akiyama*
Department of Dermatology, Hokkaido University Graduate School of Medicine, North 15 West 7, Sapporo, Hokkaido 060-8638, Japan

Toshifumi Nomura[a,b]
[a]*Department of Dermatology, Hokkaido University Graduate School of Medicine, North 15 West 7, Sapporo, Hokkaido 060-8638, Japan*

[b]*Epithelial Genetics Group, Division of Molecular Medicine, University of Dundee, Colleges of Life Sciences and Medicine, Dentistry & Nursing, Dundee, UK*

Ikue Nemoto-Hasebe
Department of Dermatology, Hokkaido University Graduate School of Medicine, North 15 West 7, Sapporo, Hokkaido 060-8638, Japan

Riichiro Abe
Department of Dermatology, Hokkaido University Graduate School of Medicine, North 15 West 7, Sapporo, Hokkaido 060-8638, Japan

W.H. Irwin McLean
Epithelial Genetics Group, Division of Molecular Medicine, University of Dundee, Colleges of Life Sciences and Medicine, Dentistry & Nursing, Dundee, UK

Hiroshi Shimizu
Department of Dermatology, Hokkaido University Graduate School of Medicine, North 15 West 7, Sapporo, Hokkaido 060-8638, Japan

*Corresponding author. Tel.: +81 11 706 7387;
fax: +81 11 706 7820
E-mail address: akiyama@med.hokudai.ac.jp
(M. Akiyama)

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Japanese-Specific Filaggrin Gene Mutations in Japanese Patients Suffering from Atopic Eczema and Asthma

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TO THE EDITOR

Mutations in *FLG*, the gene encoding profilaggrin/filaggrin, are the underlying cause of ichthyosis vulgaris (OMIM 146700) and an important predisposing factor for atopic eczema (AE) (Sandilands *et al.*, 2007). *FLG* mutations are also significantly associated with asthma with AE mainly in the European population (Rodríguez *et al.*, 2009; van den Oord and Sheikh, 2010). The presence of population-specific *FLG* mutations has been reported in both the European and Asian races (Nomura *et al.*, 2007; Sandilands *et al.*, 2007). To clarify whether *FLG* mutations are a predisposing factor for asthma in the non-European population, we initially studied 172 Japanese AE patients (mean age, 24.8 ± 9.1 years) and 134 unrelated Japanese control individuals (healthy volunteers; mean age, 27.9 ± 6.0 years). All AE patients had been diagnosed based on widely recognized diagnostic criteria (Hanifin and Rajka, 1980). The majority of AE patients and control individuals were identical to those in a previous study (Nemoto-Hasebe *et al.*, 2010). In this AE cohort, 73 AE patients (mean age, 25.4 ± 8.9 years) experienced complications with asthma. Furthermore, we studied another Japanese asthma cohort (137 patients; mean age, 58.2 ± 16.9 years). Patients were considered asthmatic based on the presence of recurrent episodes of ≥2 of the three symptoms (coughing, wheezing, or dyspnea) associated with demonstrable reversible airflow limitation, either spontaneously or with an inhaled short-acting β2-agonist and/or increased airway responsiveness to methacholine (Isada *et al.*, 2010). Fully informed consent was obtained from the participants or their legal guardians for this study. This study had been approved by the Ethical Committee at Hokkaido University Graduate School of Medicine and was conducted according to the Declaration of Helsinki Principles.

FLG mutation screening revealed that 27.4% of patients in our Japanese AE complicated with asthma case series carried one or more of the eight *FLG* mutations (combined minor allele frequency of 0.151, $n=146$) (Table 1). Conversely, 26.3% of Japanese AE patients without asthma carried one or more of the eight *FLG* mutations (combined minor allele frequency of 0.147, $n=198$). The *FLG* variants are also carried by 3.7% of Japanese control individuals (combined minor allele frequency of 0.019, $n=268$). We found that all compound heterozygous mutations were present in *trans* by observing transmission or haplotype analysis (Nomura *et al.*, 2007, 2008). There is a statistically significant association between the eight *FLG* mutations and AE with asthma, and between the eight *FLG* mutations and AE without asthma (Table 1). Moreover, AE complicated with asthma manifested in heterozygous carriers of *FLG* mutations with an odds ratio for AE and asthma of 9.74 (95% confidence interval 3.47–27.32), suggesting a relationship between *FLG* mutations and AE with asthma.

In the Japanese general asthma cohort, 8.0% of the asthma patients carried one or more of the eight *FLG* mutations (combined minor allele frequency of 0.04, $n=274$) (Table 2). Whereas, of the Japanese patients with asthma complicated by AE, 22.2% carried one or more of the *FLG* mutations (combined minor allele frequency of 0.11, $n=36$). In contrast, 5.9% of asthma patients without AE carried one or more of the *FLG* mutations (combined minor allele frequency of 0.03, $n=238$). There was a statistically significant association between the eight *FLG* mutations and asthma with AE (Table 2). There was no statistically significant association between the *FLG* mutations and entire asthma patients, nor between *FLG* mutations and asthma without AE. We cannot exclude the possibility that this lack of significant association is due to the small number of the patients included in this study. We used the same control set for both case-controlled studies. Thus, strictly speaking, there is no independent replication for the control group.

Recent meta-analysis revealed that *FLG* mutations are significantly associated with asthma in the European population and there are especially, strong effects observed for *FLG* mutations for the compound phenotype, asthma in addition to eczema (Rodríguez *et al.*, 2009; van den Oord and Sheikh, 2010). In contrast, there appeared to be no association of *FLG* mutations with asthma in the absence of eczema (Rodríguez *et al.*, 2009; van den Oord and Sheikh, 2010).

This Japanese cohort has a completely different *FLG* mutation spectrum from those in the European and the North American populations. However, our results clearly confirm the strong association of *FLG* mutations with our Japanese cohort of AE patients with asthma complications, and the association of *FLG* mutations and asthma patients with AE complications, for the first time outside Europe or North America. Conversely, this study showed no significant correlation between general asthma patients and *FLG* mutations, suggesting that atopic asthma patients associated with *FLG* mutations are a minority among general asthma patients. The frequency of heterozygous, compound heterozygous, and homozygous *FLG* mutation carriers

Abbreviation: AE, atopic eczema

Table 1. Atopic eczema case–control association analysis for *FLG* null variants in Japan

	R501X		3321delA		S1695X		Q1701X		S2554X		S2889X		S3296X		K4022X		Combined			
Genotype	Con	AE	Con	AE	Con	AE	Con	AE	Con	AE	Con	AE	Con	AE	Con	AE	Con	AE (total)	AE (asthma+)	AE (asthma−)
AA	134	172	133	163	133	172	134	169	133	162	132	152	134	166	134	169	129	126	53	73
Aa	0	0	1	9	1	0	0	3	1	10	2	20	0	6	0	3	5	41	18	23
aa	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5[1]	2	3
Total	134	172	134	172	134	172	134	172	134	172	134	172	134	172	134	172	134	172	73	99

Abbreviations: AE, atopic eczema; CI, confidence interval; Con, healthy control; OR, odds ratio.
For combined genotype: AE+asthma, exact *P*-value of Pearson χ^2-test=1.909×10^{-6}, OR and 95% CI for dominant models (AA vs aX)=9.737 (3.473–27.322); AE−asthma, exact *P*-value of Pearson χ^2-test=7.189×10^{-7}, OR and 95% CI for dominant models (AA vs aX)=9.191 (3.383–24.938); all AE, exact *P*-value of Pearson χ^2-test=1.189×10^{-7}, OR and 95% CI for dominant models (AA vs aX)=9.416 (3.625–24.450).
[1]All the five patients were compound heterozygotes for minor alleles.

Table 2. Asthma case–control association analysis for *FLG* null variants in Japan

	R501X		3321delA		S1695X		Q1701X		S2554X		S2889X		S3296X		K4022X		Combined			
Genotype	Con	Asthma	Con	Asthma	Con	Asthma	Con	Asthma	Con	Asthma	Con	Asthma	Con	Asthma	Con	Asthma	Con	Asthma (total)	Asthma (AE+)	Asthma (AE−)
AA	134	137	133	137	133	137	134	137	133	133	132	132	134	136	134	136	129	126	14	112
Aa	0	0	1	0	1	0	0	0	1	4	2	5	0	1	0	1	5	11	4	7
aa	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	134	137	134	137	134	137	134	137	134	137	134	137	134	137	134	137	134	137	18	119

Abbreviations: AE, atopic eczema; CI, confidence interval; Con, healthy control; OR, odds ratio.
For combined genotype: asthma+AE, exact *P*-value of Pearson χ^2-test=0.0122, OR and 95% CI for dominant models (AA vs aX)=7.3692 (1.7715–30.6748); asthma−AE, exact *P*-value of Pearson χ^2-test=0.5563, OR and 95% CI for dominant models (AA vs aX)=1.6124 (0.4979–5.2219); all asthma, exact *P*-value of Pearson χ^2-test=0.1968, OR and 95% CI for dominant models (AA vs aX)=2.2523 (0.7609–6.6667).

observed in our Japanese controls was only 3.7%, which was much lower than that seen in European general population, where it is approximately 7.5%. This suggested that there may be further mutations yet to be discovered in the Japanese. As we have sequenced more than 40 Japanese families with ichthyosis vulgaris, there is now little possibility that further highly prevalent mutations will be found in the Japanese population. However, it is still possible that there might be multiple, further low-frequency *FLG* mutations discovered in the Japanese population. In addition, because of the relatively small sample size of this genetic study, further replication in association studies will be required for *FLG* mutations and asthma in Japan.

In our cohorts, serum IgE levels were extremely high (median, 3141.9 IU ml^{-1}; 25th–75th percentiles, 1276.0–9753.0 IU ml^{-1}) in AE patients with asthma ($n=73$) in the AE cohort, compared with that in total asthma patients (median, 156.0 IU ml^{-1}; 25th–75th percentiles, 71.05–441.45 IU ml^{-1}, $n=137$) in the asthma cohort. These findings suggest that extrinsic allergic sensitization might have an important role in atopic asthma pathogenesis. Recent studies hypothesized skin barrier defects caused by *FLG* mutation(s) allow allergens to penetrate the skin, resulting in initiation of further immune response and leading to the development of systemic allergies, including atopic asthma (Fallon *et al.*, 2009). In patients with asthma that also harbor *FLG* mutations, we could not exclude the possibility that the systemic effects of early eczema might simply influence airway responsiveness (Henderson *et al.*, 2008).

CONFLICT OF INTEREST

Irwin McLean has filed patents relating to genetic testing and therapy development aimed at the filaggrin gene.

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Rinko Osawa[1], Satoshi Konno[2], Masashi Akiyama[1], Ikue Nemoto-Hasebe[1], Toshifumi Nomura[1,3], Yukiko Nomura[1], Riichiro Abe[1], Aileen Sandilands[3], W.H. Irwin McLean[3], Nobuyuki Hizawa[4,5], Masaharu Nishimura[2] and Hiroshi Shimizu[1]

[1]Department of Dermatology, Hokkaido University School of Medicine, Sapporo, Japan; [2]First Department of Medicine, Hokkaido University School of Medicine, Sapporo, Japan; [3]Epithelial Genetics Group, Division of Molecular Medicine, University of Dundee, Colleges of Life Sciences and Medicine, Dentistry & Nursing, Dundee, UK;

[4]*Department of Pulmonary Medicine, Institute of Clinical Medicine, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba, Ibaraki, Japan and* [5]*University Hospital, University of Tsukuba, Tsukuba, Ibaraki, Japan*
E-mail: akiyama@med.hokudai.ac.jp

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See related commentary on pg 2703

RNase 7 Protects Healthy Skin from *Staphylococcus aureus* Colonization

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TO THE EDITOR

The Gram-positive bacterium *Staphylococcus aureus* is an important pathogen that causes various skin infections (Miller and Kaplan, 2009). However, healthy skin is usually not infected by *S. aureus,* despite the high carrier rates in the normal population (Noble, 1998). This suggests that the cutaneous defense system has the capacity to effectively control the growth of *S. aureus.* There is increasing evidence that antimicrobial proteins are important effectors of the cutaneous defense system (Harder *et al.*, 2007). A recent study reported that keratinocytes contribute to cutaneous innate defense against *S. aureus* through the production of human β-defensin-3 (Kisich *et al.*, 2007). In addition to human β-defensin-3,, other antimicrobial proteins may also participate in cutaneous defense against *S. aureus.* One candidate is RNase 7, a potent antimicrobial ribonuclease that is highly expressed in healthy skin (Harder and Schröder, 2002; Köten *et al.*, 2009).

To investigate the hypothesis that RNase 7 may contribute to protect healthy skin from *S. aureus* colonization, we first incubated natural RNase 7 isolated from stratum corneum skin extracts (Harder and Schröder, 2002) with *S. aureus* (ATCC 6538). In concordance with our initial report about RNase 7 (Harder and Schröder, 2002), we verified that RNase 7 exhibited a high killing activity against *S. aureus* (lethal dose of 90% $= 3–6\,\mu g\,ml^{-1}$).

Recently, we reported a moderate induction of RNase 7 mRNA expression in primary keratinocytes treated with heat-killed *S. aureus* (Harder and Schröder, 2002). To assess the induction of RNase 7 by *S. aureus* in the

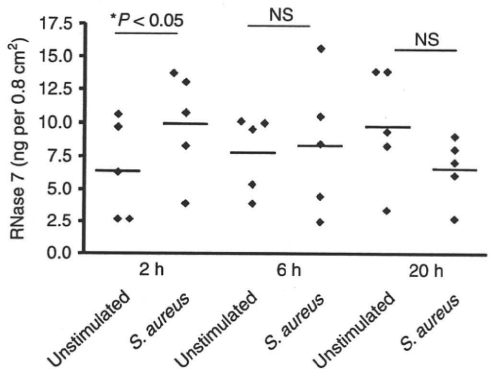

Figure 1. Induced secretion of RNase 7 on the skin surface on treatment with living *S. aureus*. Defined areas (0.8 cm^2) of skin explants derived from plastic surgery were incubated with or without approximately 1,000 colony-forming units of *S. aureus* (ATCC 6538) in 100 µl of sodium phosphate buffer. After 2, 6, and 20 hours, the concentration of secreted RNase 7 was determined by ELISA. Stimulation with *S. aureus* for 2 hours revealed a significant induction as compared with the unstimulated control after 2 hours (*$P < 0.05$, Student's *t*-test; n.s. = not significant). Data shown are means of triplicates of five skin explants derived from five donors.

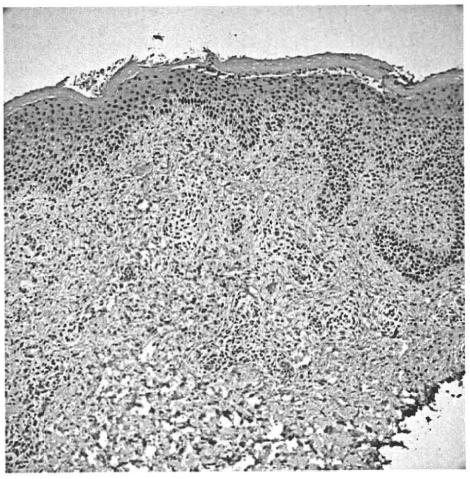

Fig 2. Immunohistochemistry of skin for angiopoietin-2. Note the abundant staining in the basal epidermis and dermal inflammatory cells. (Original magnification: ×100.)

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Reprint requests: Jack L. Arbiser, MD, PhD, Department of Dermatology, Emory University School of Medicine, WMB 5309, 1639 Pierce Dr, Atlanta, GA 30322.

E-mail: jarbise@emory.edu

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Aleukemic leukemia cutis with extensive bone involvement

To the Editor: Aleukemic leukemia cutis (ALC) is a rare condition that is characterized by the invasion of leukemic cells into the skin before such cells are observed in the peripheral blood.[1] We present a case of ALC with multiple bone metastases.

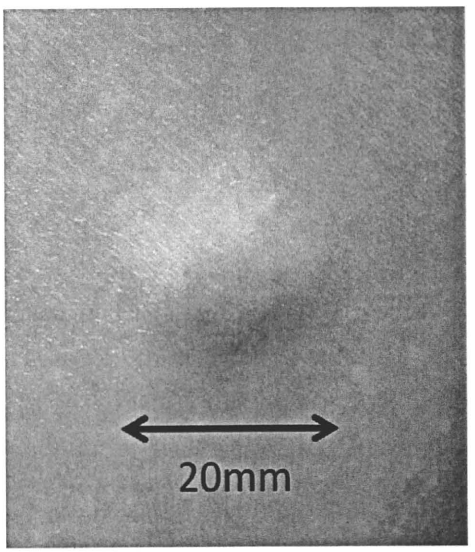

Fig 1. A slightly violaceous nodule on the middle aspect of the left thigh.

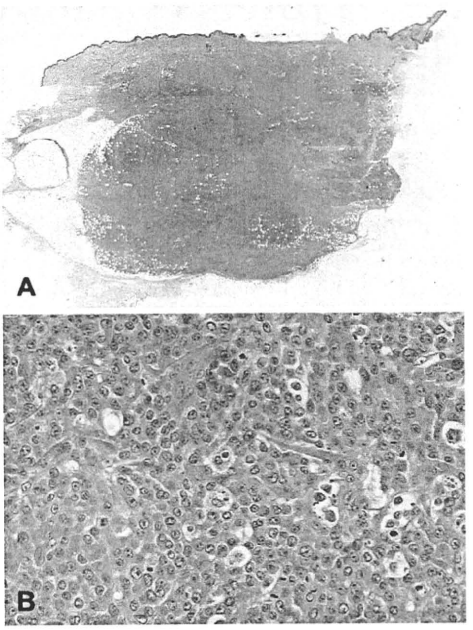

Fig 2. Skin biopsy findings. **A**, Dense, nodular, diffuse infiltrate of monotonous uniform cells involving the dermis and subcutaneous fat. **B**, A nodule of cells with round nuclei; prominent single or multiple nucleoli; abundant pale, slightly eosinophilic cytoplasm; and a number of atypical mitotic figures.

An 81-year-old man had a 2-month history of asymptomatic nodules on his trunk and legs. The physical examination revealed five subcutaneous nodules measuring up to 10 mm in size on his back and legs and a firm, slightly violaceous nodule

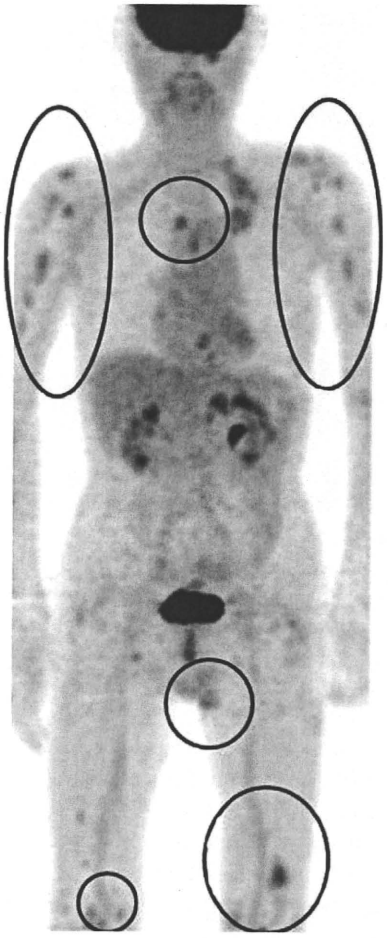

Fig 3. A positron emission tomography scan reveals extensive high-density areas in the bone and in the subcutaneous tissue of the trunk and the extremities, suggesting multiple metastases.

measuring 20 mm in diameter on the middle aspect of his left thigh (Fig 1). A complete blood cell count and chemical analysis showed no pathologic changes. Histopathologic examination of a skin biopsy specimen revealed a dense, nodular, diffuse infiltrate of monotonous uniform cells with round nuclei, prominent single or multiple nucleoli, and abundant pale, slightly eosinophilic cytoplasmic cells throughout the dermis and subcutaneous fat (Fig 2, *A*). A number of atypical mitotic figures were seen (Fig 2, *B*). The tumor cells were positive for leukocyte common antigen, CD68, and myeloperoxidase. A histologic diagnosis of myeloid leukemia cutis with possible monocytic lineage was made. However, bone marrow aspiration showed neither an increase in blasts nor abnormal cell infiltration, and repeated peripheral blood cell counts were normal, with no atypical cells. A diagnosis of ALC was established. Positron emission tomography (PET) revealed extensive high-density areas in the bone and subcutaneous tissue, suggesting multiple metastases (Fig 3). Seven weeks after his first visit, a peripheral blood cell examination disclosed 8% atypical monocytic cells, suggesting a diagnosis of acute myeloid leukemia. The patient refused other studies and died 1 week later.

ALC is a rare form of leukemia with a poor prognosis. The term "aleukemic" has been used to designate a form of leukemia in which there are no leukemic cells in the blood.[2] ALC precedes peripheral blood or bone marrow abnormalities at least 1 month before the systemic findings. Once leukemic cells appear in the peripheral blood or bone marrow, the mean survival time ranges from 3 to 30 months.[3,4] The clinical features of ALC include multiple papules, nodules, or infiltrated plaques with a red-brown or plum-colored surface. Histologic findings show the infiltration of leukemic cells in the dermal or subcutaneous tissues. The cytologic features of the tumor cells include large, vesicular nuclei and multiple prominent nucleoli.[3] Because of the rarity of the disease, there is no consensus on the treatment of choice for ALC; radiotherapy, chemotherapy, and total body electron therapy have achieved variable results.[1,3-8] A study by Chang et al[4] of a large group of ALC patients showed that the most common extramedullary site of involvement after the skin (31 of 31 patients) was the lymph nodes (8 of 31 patients) followed by the spleen (2 of 31 patients). Although no reports of a clinical presentation of ALC with multiple sites of bone infiltration were found in a thorough search of the English-language literature, extramedullary leukemia is known to occur in bone.[5] We emphasize that the routine assessment of a patient with ALC should include systemic investigations such as PET, taking into consideration the possibility of bone involvement.

Maria Maroto Iitani, MD, MSc,[a] Riichiro Abe, MD, PhD,[a] Teruki Yanagi, MD,[a] Asuka Hamasaka, MD, PhD,[a] Yasuki Tateishi, MD, PhD,[a] Yukiko Abe, MD, PhD,[a] Miki Ito, MD,[b] Takeshi Kondo, MD, PhD,[c] Kanako Kubota, MD, PhD,[d] and Hiroshi Shimizu, MD, PhD[a]

Departments of Dermatology, Hokkaido University Graduate School of Medicine[a] and the National Hospital Organization Hokkaido Cancer Center[b]; Departments of Gastroenterology and Hematology,[c] Hokkaido University Graduate School of Medicine; and the Department of Surgical Pathology,[d] Hokkaido University Hospital, Sapporo, Japan

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Reprint requests: Maria Maroto Iitani, MD, MSc, Department of Dermatology, Hokkaido University Graduate School of Medicine, North 15, West 7, Kita-ku, Sapporo 060-8638, Japan.

E-mail: miitani@med.hokudai.ac.jp

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Onychopapilloma presenting as longitudinal leukonychia

To the Editor: Onychopapilloma is an uncommon benign nail neoplasm characterized histologically by distal subungal hyperkeratosis and nail matrix metaplasia of the nail bed with marked papillomatosis. The majority of cases present clinically as localized longitudinal erythronychia. We report a case of onychopapilloma presenting as localized longitudinal leukonychia.

A 50-year-old woman was referred for the evaluation of dystrophy of the right third fingernail. The nail plate had split distally for several years. Her medical history was noncontributory. The physical examination revealed a 1-mm wide band of longitudinal leukonychia with a slight longitudinal ridge on the right third fingernail. No erythronychia was present. Distally, there was a V-shaped notch and split, with a keratotic 1-mm papule at the hyponychium (Fig 1). The other nails were normal. Lateral nail plate curl avulsion exposed a longitudinal ridge extending from the midmatrix onto the nail bed. A longitudinal biopsy from matrix to hyponychium was performed. On histologic examination, the nail bed exhibited slender, elongated, and hyperplastic rete ridges with underlying fibrosis and thickening of the fibrovascular dermal stroma. Upper nail bed keratinocytes were large and exhibited ample pink cytoplasm similar to the nail matrix keratogenous zone. Hyperkeratosis was seen at the hyponychium (Fig 2). A periodic acid–Schiff test did not reveal fungal elements. These findings were consistent with the diagnosis of onychopapilloma.

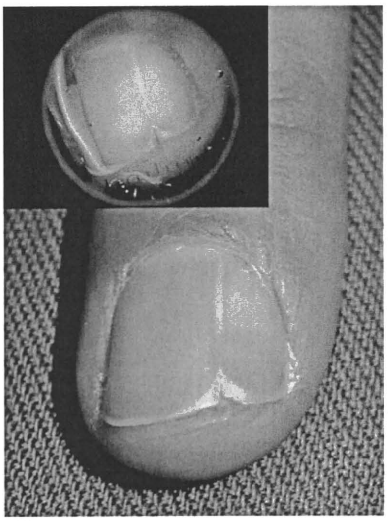

Fig 1. Fingernail with a 1-mm wide band of longitudinal leukonychia and distal V-shaped splitting, onycholysis, and a keratotic papule at the free edge of the nail. Dermatoscopy highlights these findings (*inset*).

Onychopapilloma was first reported in 1995 by Baran and Perrin,[1] who described four cases of "distal subungual keratosis with multinucleate cells." The term "onychopapilloma" was later coined in 2000 when the authors reported a second series of 14 cases with similar clinical and histopathologic features.[2] Key among these features were the upper cell layers in the nail bed epithelium exhibiting abundant eosinophilic cytoplasm resembling the nail matrix keratogenous zone, and was thought to indicate matrix metaplasia of the nail bed epithelium. Additional findings included acanthosis and papillomatosis of the distal nail bed epithelium. Multinucleated cells were found variably. In both series, all lesions presented as either longitudinal erythronychia or longitudinal bands of splinter hemorrhages, several of which were associated with distal onycholysis. Other occurrences of suspected onychopapilloma have been reported, including one case representing solitary nail bed lichen planus, and also in the spectrum of localized longitudinal erythronychia.[3] In addition to onychopapilloma, the differential diagnosis for localized longitudinal erythronychia includes Bowen disease,[2,3] and histologic investigation is often warranted.

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Donor Pretreatment with DHMEQ Improves Islet Transplantation

Tohru Takahashi, M.D.,*,[1] Shuichiro Matsumoto, M.D., Ph.D.,* Michiaki Matsushita, M.D., Ph.D.,* Hirofumi Kamachi, M.D., Ph.D.,* Yosuke Tsuruga, M.D., Ph.D.,* Hironori Kasai, M.D.,* Masaaki Watanabe, M.D.,* Michitaka Ozaki, M.D.,† Hiroyuki Furukawa, M.D., Ph.D.,* Kazuo Umezawa, Ph.D.,‡ and Satoru Todo, M.D., Ph.D.*

**Department of General Surgery, Hokkaido University Graduate School of Medicine, Sapporo, Hokkaido, Japan; †Department of Molecular Surgery, Hokkaido University Graduate School of Medicine, Sapporo, Hokkaido, Japan; and ‡Department of Applied Chemistry, Faculty of Science and Technology, Keio University, Yokohama, Kanagawa, Japan*

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Background. **Currently, pancreatic islet transplantation to achieve normoglycemia in insulin-dependent diabetes mellitus (IDDM) requires two or more donors. This may be due to the inability to transplant functionally preserved and viable islets after isolation. Islets have already been subjected to various harmful stresses during the isolation process leading to apoptosis. One of the intracellular signaling pathways, the transcription factor nuclear factor-κB (NF-κB)-related pathway, is relevant to the mechanism of β-cell apoptosis in isolated islets. We attempted to prevent islet apoptosis during isolation by a novel NF-κB inhibitor, dehydroxymethylepoxyquinomicin (DHMEQ).**

Materials and Methods. **DHMEQ was injected intraperitoneally into donor mice 2 h prior to isolation. NF-κB activation, the functioning of isolated islets, apoptosis after isolation, and cytokine- and apoptosis-related genes were analyzed. After 160 equivalents of islets were transplanted into diabetic mice, graft survival and function were evaluated.**

Results. **Intra-islet NF-κB was activated immediately after isolation, and DHMEQ inhibited NF-κB activation without deterioration of islet function. DHMEQ significantly prevented apoptosis by inhibiting caspase 3/7 activities and down-regulated Bax, a pro-apoptotic gene. Donor pretreatment with DHMEQ significantly improved engraftment in syngeneic islet transplantation in mice, thus preserving insulin contents in the graft liver, as assessed by functional and histologic analyses.**

Conclusions. **DHMEQ is a promising agent in islet transplantation because it protects islets from apoptosis during isolation stress. Donor pretreatment with DHMEQ can significantly affect the success of islet engraftment.**

Key Words: **islet transplantation; donor pretreatment; DHMEQ; NF-κB inhibitor; NF-κB; islet isolation; apoptosis; mouse.**

INTRODUCTION

Islet transplantation for insulin-dependent diabetes mellitus (IDDM) by the Edmonton protocol has been highly successful, but this therapeutic approach has a major limitation in that islets from two to four cadaveric pancreases are required to achieve insulin independence in one diabetic recipient [1]. Recently, the reported success of human islet transplantation has further emphasized the imbalance between the supply and demand of islet tissues for transplantation [2]. The viability of islets is lost in the course of procurement to transplantation [3]. Therefore, the prevention of islet loss is important for improving engraftment and for reducing the number of islets required to achieve normoglycemia in diabetes. This, in turn, will allow us to optimize the number of transplants that can be performed given the current limited supply of islets. To define strategies to preserve the transplanted β-cell mass, it is essential to know the extent of β-cell loss and to identify the mechanisms underlying β-cell destruction and protection.

Islets have already been susceptible to various harmful stresses, such as ischemia, disruption from the extracellular matrix due to preservation, digestion,

[1] To whom correspondence and reprint requests should be addressed at Department of General Surgery, Hokkaido University Graduate School of Medicine, North 14 West 5, Kitaku, Sapporo, Hokkaido 060-8648, Japan. E-mail: Ttohru616@aol.com.

0022-4804/$36.00

isolation, and culture. These procedures are thus expected to lead islets to apoptosis, which would be the major cause of islet loss [3, 4]. Although the mechanism underlying β-cell apoptosis after isolation is not clearly understood, the transcription factor nuclear factor-κB (NF-κB) [5, 6] is considered to be involved in β-cell apoptosis.

NF-κB is a family of inducible transcription regulatory proteins expressed in a variety of tissues [7]. Some studies have reported that NF-κB was upregulated in isolated islets and that cytokines such as IL-1β, IFNγ, and TNFα were produced from them by NF-κB activation. Then, intra-islet cytokine production [8] can further aggravate the apoptotic pathway. On the other hand, NF-κB also regulates pro- [9, 10] or anti-apoptotic genes [11]. Hence whether NF-κB has an inducible or inhibitory effect on apoptosis is a matter of controversy. However, many recent studies have verified that NF-κB inhibition protects insulin-secreting cell lines or primary islets from cytokine-induced apoptosis [6, 12, 13].

A novel NF-κB inhibitor, dehydroxymethylepoxyquinomicin (DHMEQ), is a 5-dehydroxymethyl derivative of epoxyquinomicin C, an antibiotic originally isolated from *Amycolatopsis* sp [14] that has been found to inhibit NF-κB activation at the level of nuclear translocation [15]. DHMEQ can abrogate constitutive NF-κB activity and inhibit the production of proinflammatory cytokines from various cells [16, 17]. These findings suggest that DHMEQ may be a new therapeutic agent for inflammatory diseases.

In the current study, we attempted to inhibit intraislet NF-κB activation during isolation by DHMEQ. We aimed to prevent islet apoptosis by inhibiting inflammatory cytokines or by regulating apoptosis-related genes and to preserve the islet's ability to release insulin.

MATERIALS AND METHODS

Animals

Male C57BL/6J mice, aged 9–12 wk, were purchased from Clea Japan, Inc. (Tokyo, Japan) and used as donors and recipients. All studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals at Hokkaido University Graduate School of Medicine.

Islet Isolation and Culture

DHMEQ, dissolved in 2.4% dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO) and 0.5% carboxymethylcellulose (CMC) (Sigma) or vehicle (DMSO with CMC) was given by intraperitoneal injection (i.p.) to donors 2 h before isolation. DHMEQ was administered at 12 mg/kg. Pancreatic islets were distended by intraductal injection of collagenase (2 mg/mL; Worthington Biochemical, Lakewood, NJ), and digestion was performed at 37°C for 20 min. The islets were separated on Ficoll (Sigma) density gradients by centrifugation at 2200 rpm for 10 min, and the isolated islets were counted under a scaled microscope at 40× to calculate islet equivalents (IEQ). One IEQ was the islet tissue mass equivalent to a spherical islet 150 μm in diameter. Islets were hand-picked individually under the microscope to ensure pure islet preparations. The islets were cultured in RPMI-1640 (Sigma) medium supplemented with 10% FCS (Gibco, Long Island, NY) and antibiotic antimycotic solution (100 units/mL penicillin, 0.1 mg streptomycin, and 0.25 μg amphotericin B) (Sigma) in a 5% CO_2 humidified atmosphere at 37°C.

Analysis of NF-κB Transcriptional Activity

Islets treated with vehicle or DHMEQ were isolated, and nuclear proteins were extracted 30 min after isolation by using NE-PER nuclear and cytoplasmic extraction reagents (Pierce Chemical Co., Rockford, IL) according to the manufacturer's instructions. Protein concentrations were determined by the BCA Protein Assay Reagent Kit (Pierce Chemical Co.). NF-κB DNA-binding activity in the nuclear extract (NE) was measured using the Trans-AM NF-κB p65 enzyme-linked immunosorbent assay (ELISA) kit (Active Motif Japan, Tokyo, Japan) according to the manufacturer's instructions. Briefly, 1.5 μg NE was added to a 96-well plate, to which an oligonucleotide-containing NF-κB consensus-binding site had been immobilized. The NF-κB complex bound to the oligonucleotide was detected by adding a specific monoclonal antibody (mAb) to a p65 subunit. A secondary horseradish peroxidase (HRP)-conjugated mAb was added and developed with a tetramethylbenzidine substrate. After an optimal development time, the reaction was stopped using H_2SO_4 0.5 mol/L, and absorbance was measured by 450 nm. The specificity of the assay was monitored by competition experiments using NF-κB wild-type and mutant consensus oligonucleotides provided in the kit. The activated form of NF-κB exists only in the nuclear fraction, and this kit detects only this activated form. NF-κB activation in three different nuclear extracts ($n = 3$) was checked by comparison with the NF-κB level of cytosol protein, positive control (PC), and negative control (NC) provided in the kit, which were simultaneously measured.

Static Glucose-Stimulated Insulin Secretion (GSIS)

After islets were cultured at 37°C for 12 or 24 h, 20 islets were hand-picked and incubated for 60 min in RPMI-1640 with 10% FCS containing 1.67 mM glucose. Thereafter, the solution was changed and the islets were incubated for 60 min with 0.5 mL of 1.67 mM glucose, then 16.7 mM glucose in RPMI-1640 with 10% FCS at 37°C. Supernatants were collected for baseline and stimulated insulin release, and were stored at −20°C. Subsequently, the insulin contents of the supernatants ($n = 6$) were analyzed using ELISA (Shibayagi, Gunma, Japan). A stimulation index was calculated by dividing the total insulin amount released from islets cultured in the high-glucose medium by the total insulin amount released from islets cultured in the low-glucose medium.

Real-Time PCR for Cytokine- and Apoptosis-Related Gene Expression in Isolated Islets

Total RNA was isolated from mouse primary islets using the Qiagen RNeasy kit (Qiagen, Valencia, CA). cDNA was reverse-transcribed using Omniscript (Qiagen) for 60 min at 37°C. Amplification of resultant cDNA was performed by PCR with TAKARA Ex Taq (Takara Bio, Ohtsu, Japan). To measure murine monocyte chemoattractant protein-1 (MCP-1), IL-1β, Bax, Bcl-2, and β-actin expression, real-time PCR was performed using an ABI-Prism 7000 sequence detector (Applied Biosystems, Foster City, CA). The PCR reaction was performed in a 25 μL reaction volume that contained 1 μL (100 ng) of the template cDNA, 200 nM each of the sense and antisense primers, and 2× Power SYBR Green PCR Master Mix (Applied Biosystems) in duplicate. PCR amplification profiles included an initial incubation at 50°C for 2 min, denaturation at 95°C for 5 min, and 40 cycles at 95°C for 30 s and 60°C for 1 min. Primer sequences (Invitrogen, Carlsbad,

TABLE 1

Primer Sequences of Real-Time PCR and Product Size

Gene	Sequences	Size (bp)
MCP-1	forward 5′-ACCTGCTGCTACTCATTCACC-3′ reverse 5′-CATTCCTTCTTGGGGTCAG-3′	149
IL-1β	forward 5′-AGTTGACGGACCCCAAAAG-3′ reverse 5′-GTGATACTGCCTGCCTGAAG-3′	125
Bax	forward 5′-TGGAGATGAACTGGACAGCA-3′ reverse 5′-GAAGTTGCCATCAGCAAACA-3′	118
Bcl-2	forward 5′-AGTACCTGAACCGGCATCTG-3′ reverse 5′-CATGCTGGGGCCATATAGTT-3′	82
β-actin	forward 5′-CTGTATTCCCCTCCATCGTG-3′ reverse 5′-AATGGGGTACTTCAGGGTCA-3′	128

CA) and the sizes of the fragments generated by PCR reactions are shown in Table 1.

The relative standard curve method was used to quantify mRNA levels against a murine cell line, RAW264.7. A standard curve was developed based on the principle that a plot of the log of the diluted cDNA contents of the standard *versus* the threshold cycle results in a straight line. The level of sample mRNA was normalized for β-actin as an internal control.

Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine Triphosphate Nick End Labeling (TUNEL) Assay

Donor-pretreated islets with or without DHMEQ were incubated for 48 h and fixed overnight in a solution of 4% formaldehyde (Sigma). The islets were embedded in paraffin, and 5 μm sections were deparaffinized, rehydrated, and incubated with proteinase K at room temperature for 15 min. They were then stained by the TUNEL method using the *in situ* Apoptosis Detection Kit (Takara Bio). The final reaction product was visualized by 3,3′-diaminobenzidinetetrahydrochloride (Sigma). To calculate an apoptotic index, the number of cells positive for the TUNEL reaction was determined and expressed as a percentage of the total number of cells counted. Sections were prepared from three different isolations in each group. Five fields, each containing at least 600 cells, were analyzed at 200× magnification per section, and 15 islets of 150 μm mean diameter for each counting were evaluated.

Caspase 3/7 Activity

Isolated islets were cultured in a 35 mm dish for floating cells containing RPMI-1640 medium. The islets were cultured for 24 or 48 h at 37°C, 5% CO_2. After incubation, 100 islets were hand-picked and transferred to a 1.5 mL Eppendorf tube. The tubes were centrifuged, and the islets were resuspended in 100 μL of RPMI-1640. Caspase-3 activity was measured using the Caspase-Glo 3/7 Assay (Promega Corp., Madison, WI) according to the manufacturer's instructions. Islets were lysed using the Caspase-Glo 3/7 reagent and incubated at room temperature for 1 h. Luciferase activity was measured using a GloMax 20/20 n luminometer (Promega) ($n = 6$).

Islet Transplantation

Male C57BL/6J mice were rendered diabetic by an i.p. of 180 mg/kg streptozotocin (STZ) (Sigma) freshly dissolved in citrate buffer (pH 4.5) and transplanted 7 d after STZ administration. Diabetes was considered to be present when nonfasting blood glucose levels were >450 mg/dL in two consecutive measurements. DHMEQ was given i.p. into donors 2 h prior to isolation of pancreatic islets. For each animal, 160 IEQs were transplanted *via* the portal vein; control ($n = 16$), DHMEQ ($n = 12$). Blood glucose levels were measured to evaluate graft function during the 60-day post-transplantation period. Normoglycemia was considered to be present when blood glucose levels were <200 mg/dL for two consecutive days.

Intraperitoneal Glucose Tolerance Test (IPGTT)

IPGTT was performed in transplanted mice (control: $n = 12$, DHMEQ: $n = 12$) with pretreated donor islets on postoperative day 28, and nontransplanted age-matched C57BL/6J mice ($n = 8$) served as controls. The mice were fasted for 6 h and given i.p. with 20% glucose at a dose of 2 g/kg BW. The animals were then monitored for blood glucose levels at 0, 30, 60, 90, 120, and 180 min after the injection. The area under the curve (AUC) for the IPGTT was evaluated. During the IPGTT, care was taken not to cause any unnecessary stress in the animals, and they had free access to water.

Insulin Content of Islet-Transplanted Graft Liver

The whole liver was extracted, suspended in 2 mL of distilled water, sonicated for 30 s, and added to more distilled water to a total of 3 mL, then resuspended in 7 mL of acid/ethanol (0.18 mol/L HCl in 99.5% ethanol). Insulin was extracted overnight at 4°C. Tubes were centrifuged at 3000 rpm for 10 min at 4°C, and the supernatant was stored at –20°C for future insulin determination. After neutralization, the insulin content in the extracts was measured by ELISA (control: $n = 13$, DHMEQ: $n = 11$).

Histological Analyses of Islet-Transplanted Graft Liver

Graft livers were removed 24 h after islet transplantation, fixed in 4% formalin for 24 h, and embedded in paraffin for hematoxylin-eosin (HE) and TUNEL staining. Serial-matched paraffin sections were used for these stainings. The sections were then immunostained for insulin using a guinea pig polyclonal anti-insulin primary antibody (DAKO, Glostrup, Denmark) and alkaline phosphatase-conjugated AffiniPure donkey anti-guinea pig IgG secondary antibody (Jackson Immuno-Research, West Grove, PA). The area of islet necrosis assessed by HE sections was measured by Image J software (NIH, Bethesda, MD) and was expressed as a percentage of whole islet tissue. Three mice were transplanted for each group as three independent experiments, and 8.6 islets per graft at the mean number were assessed.

Statistical Analysis

Results are presented as means ± standard deviation (SD). Statistical analyses were performed using Student's t-test and the Mann-Whitney t-test where appropriate. Differences in the duration of graft survival between groups were evaluated with the Kaplan-Meier log-rank test. A P value < 0.05 was considered statistically significant.

RESULTS

Assessment of Molecular and Morphologic Changes in Islet During Isolation Procedure—

NF-κB Activation and Effect of DHMEQ During Isolation Procedure

The level of NF-κB in NE in either group was higher than that of cytosol protein, and that of cytosol protein was nearly the same as that of NC. This means intraislet NF-κB was activated immediately after isolation. The level of NF-κB in the DHMEQ-treated group was

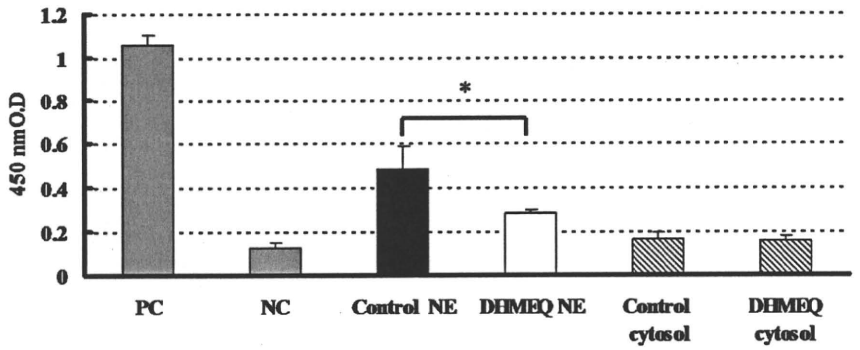

FIG. 1. NF-κB (p65) activation levels immediately after isolation. All data are representative of three independent experiments and are expressed as means ± standard deviation (SD) ($n = 3$). PC = positive control; NC = negative control; NE = nuclear extract. Intraislet NF-κB was activated immediately after isolation, and the level of NF-κB in the DHMEQ-treated group was significantly lower than that of the control. *$P < 0.05$ *versus* control.

significantly lower than that of the control ($P < 0.05$) (Fig. 1). DHMEQ significantly inhibited NF-κB activation in the isolated islets.

GSIS

The responses to low glucose are almost the same and did not decline during culture in either group. The responses to high glucose were almost the same, but declined slightly during culture in both groups (Fig. 2A). So, the stimulation indices of both groups showed a rapid drop during culture (Fig. 2B). The reduction rates of stimulation indices in the control and DHMEQ-treated groups from 12 to 24 h were 47.8% and 25.0%, respectively. Although stimulation indices of the DHMEQ-treated group look well preserved during culture, there was no significant difference among groups.

Cytokine Expression in Isolated Islets

The MCP-1 mRNA expression levels of the DHMEQ-treated group at 6 h were slightly lower than those of the control, but no significant differences were observed (Fig. 3A). The IL-1β mRNA levels of the control and DHMEQ-treated groups were 0.079 ± 0.011 and

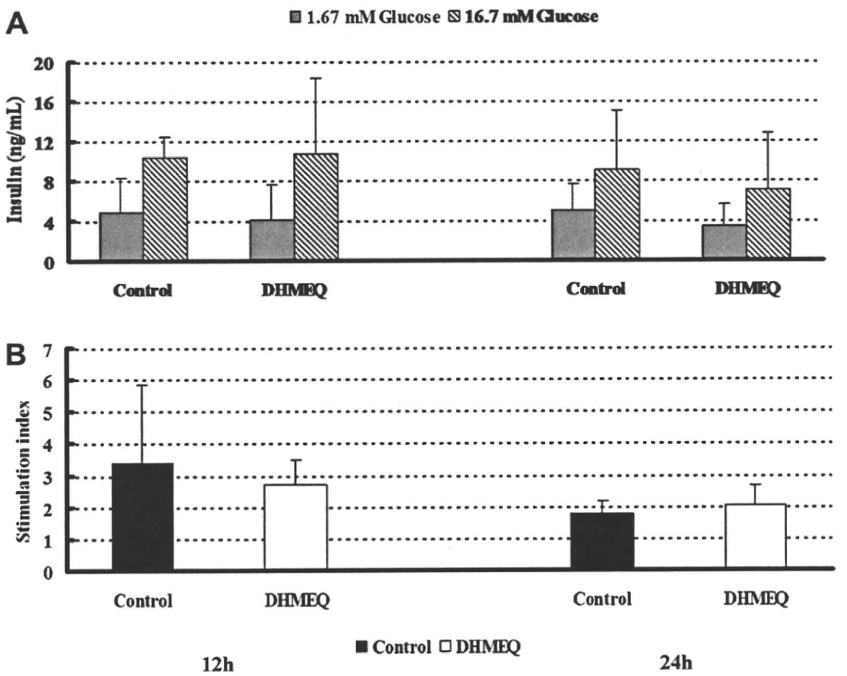

FIG. 2. GSIS. The raw data (A) and a stimulation index (B) are representative of six independent experiments, and are expressed as means ± SD ($n = 6$). There was no significant difference between the control and DHMEQ-treated groups.

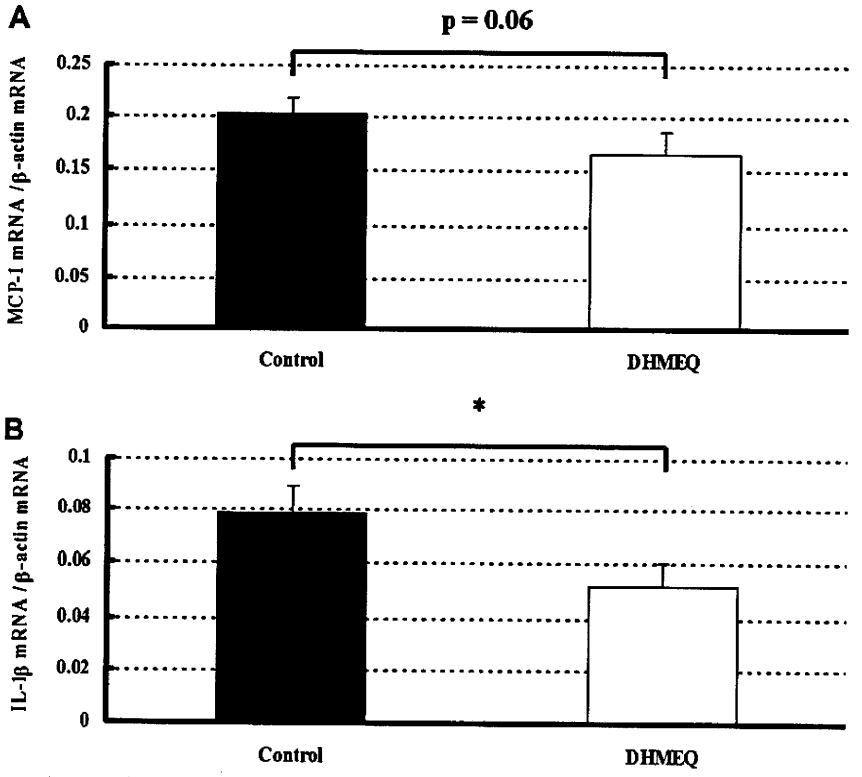

FIG. 3. Cytokine expression in isolated islets. (A) The MCP-1 expression in the DHMEQ-treated group at 6 h after isolation was lower than that of the control group, but significant differences were not observed ($P = 0.06$). (B) The IL-1β expression in the DHMEQ-treated group at 6 h after isolation was significantly lower than that of the control. Results are representative of three independent experiments and are expressed as means ± SD ($n = 3$). *$P < 0.05$ *versus* control.

0.052 ± 0.008 at 6 h, respectively, and those of the DHMEQ-treated group were significantly inhibited compared to those of the control ($P < 0.05$) (Fig. 3B).

Bax and Bcl-2 Expression in Isolated Islets

The Bax expression of the DHMEQ-treated group (2.27 ± 0.27) was significantly lower than that of the control (2.88 ± 0.24) at 6 h after isolation ($P < 0.05$) (Fig. 4A). There were no significant differences in Bcl-2 expression between the groups. DHMEQ did not inhibit Bcl-2 expression (Fig. 4B).

TUNEL Assay

The apoptotic indices of the DHMEQ-treated group (2.97% ± 2.59%) were significantly lower than those of the control (8.12% ± 5.18%) at 48 h after isolation ($P < 0.01$) (Fig. 5A). The representative islets of the control (Fig. 5B) and DHMEQ-treated groups (Fig. 5C) were displayed.

Caspase 3/7 Activity

The caspase 3/7 activities of control after isolation were gradually up-regulated, but those of the DHMEQ-treated group were constant. The activities of the DHMEQ-treated group at 24 h (1,265,812 ± 158,922 RLU) ($P < 0.05$) and 48 h after isolation (1,162,761 ± 242,885 RLU) ($P < 0.01$) were significantly lower than those of the control (1,565,278 ± 354,297 RLU at 24 h and 1,998,846 ± 378,921 RLU at 48 h, respectively) (Fig. 6).

Effect of DHMEQ Treatment on Donor After Syngeneic Intraportal Islet Transplantation

Islet Graft Survival

The blood glucose levels of the control were almost always high during the first 60 post-operative d, but in a few mice, blood glucose levels did not decrease until 4 wk after transplantation and reached normoglycemia thereafter ($n = 16$) (Fig. 7A). However, almost all mice in the DHMEQ-treated group (Fig. 7B) reached normoglycemia earlier than 14 days after transplantation ($n = 12$). The reversal rates of hyperglycemia at 60 days in the DHMEQ-treated group (83.3%) were significantly higher than those in the control group (31.2%) ($P < 0.01$) (Fig. 7C).

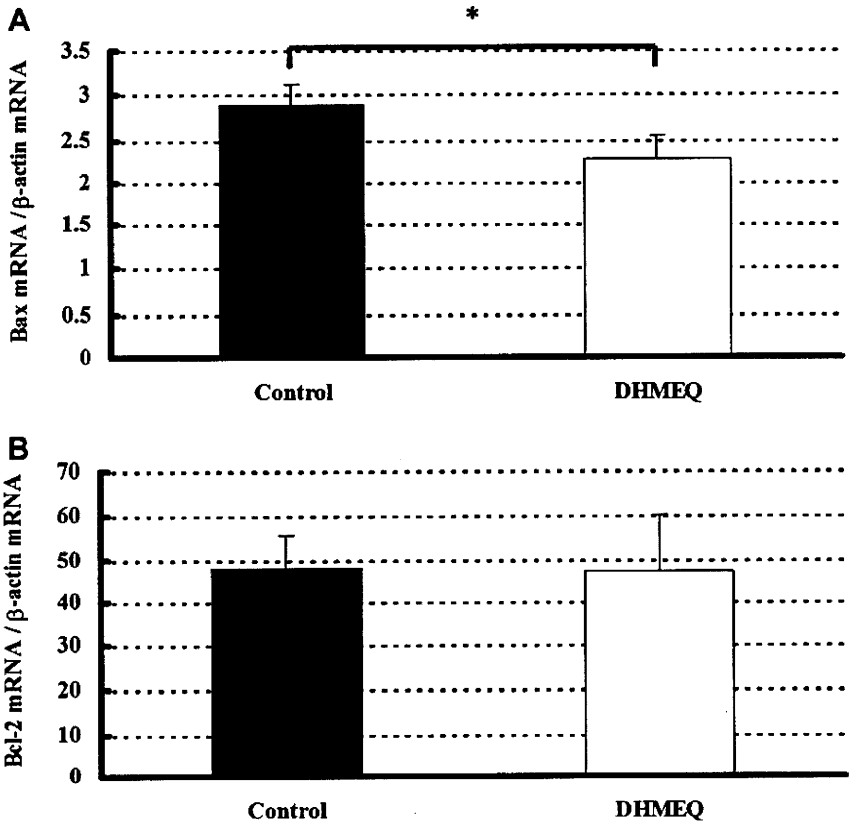

FIG. 4. Apoptosis-related gene expression in isolated islets. (A) Bax expression in the DHMEQ-treated group at 6 h after isolation was significantly lower than that of the control. (B) Bcl-2 expression in the isolated islets. No significant differences were observed between the groups. Results are representative of three independent experiments and are expressed as means ± SD ($n = 3$). *$P < 0.05$ *versus* control.

IPGTT

The time courses of blood glucose levels after intraperitoneal glucose loading in the DHMEQ-treated group were similar to those of normal mice, but the DHMEQ groups responded less than the normal mice did. The control group barely responded to glucose loading, and blood glucose levels were higher than those of the DHMEQ-treated group throughout the experiment. The blood glucose levels of the DHMEQ-treated group at 120 min (349.4 ± 96.0 mg/dL) and 180 min (234.8 ± 106.1 mg/dL) were significantly lower than those of the control group (462.0 ± 99.7 mg/dL) ($P < 0.05$), (415.9 ± 148.7 mg/dL) ($P < 0.01$), respectively (Fig. 8A). The AUCs for IPGTT of the DHMEQ-treated group (1161.5 ± 240.5) were significantly lower than those of the control (1422.7 ± 266.3) ($P < 0.05$) (Fig. 8B).

Insulin Content of Islet-Transplanted Graft Liver

Insulin contents per graft in the control and DHMEQ-treated groups were 1194.49 ± 1206.93 ng/graft and 2485.05 ± 1481.06 ng/graft, respectively. The insulin contents of the DHMEQ-treated group were significantly higher than those of the control group ($P < 0.05$) (Table 2).

Histological Analyses of Islet-Transplanted Graft Liver

The necrotic areas in the DHMEQ-treated group (26.2% ± 26.8%) (Fig. 9B) were significantly smaller than those of the control group (77.6% ± 28.7%) ($P < 0.01$) (Fig. 9A). Insulin secretory granules with many apoptotic cells were noted in the control group (Fig. 9C), but the islets of the DHMEQ-treated group had distinguished features with well-preserved nuclei, numerous secretory granules, and no apoptotic cells (Fig. 9D).

DISCUSSION

Islets must be present in sufficient numbers in order to succeed with single-donor islet transplantation, but a significant portion of the islet mass is lost during pancreatic procurement, storage, and isolation. Although apoptosis is considered the major cause of islet loss [3, 4], the mechanism underlying β-cell apoptosis in

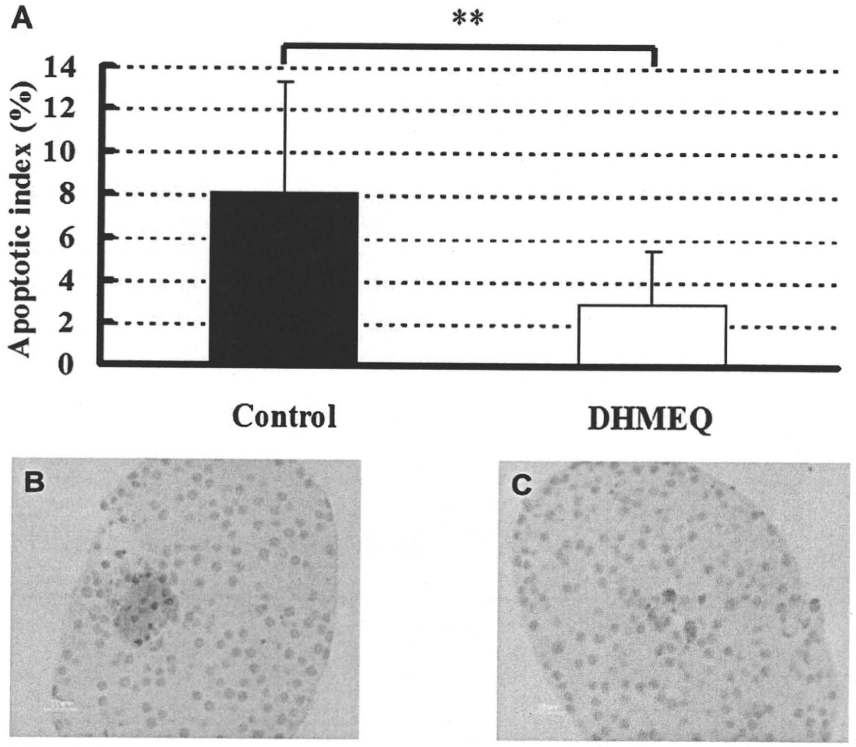

FIG. 5. Apoptosis in isolated islets and anti-apoptotic effect by DHMEQ. (A) Apoptotic index in the DHMEQ-treated group was significantly lower than that of the control. $^{**}P < 0.01$ *versus* control ($n = 15$). (B), (C) The representative of TUNEL staining in the control (B) and DHMEQ-treated group (C) was expressed (×200). (Color version of figure is available online.)

isolated islets is not clearly understood. Islet apoptosis occurs during the isolation process, which exposes islets to mechanical, enzymatic, osmotic, and ischemic stresses, leading to the disruption of the cell-matrix relationship [4, 18]. In addition, a nonspecific inflammatory reaction at the transplantation site could lead to the release of proinflammatory cytokines and free radicals, potential inducers of apoptosis [19]; and intra-islet cytokine production can exacerbate the apoptotic pathway [8]. These results indicate that islet apoptosis can cause primary nonfunction or early graft failure. So, inhibition of apoptosis before transplantation could lead to the improvement of engraftment [20]. When islets are isolated or transplanted *via* the portal vein, nuclear transcription factor NF-κB [21, 22] is upregulated and considered a contributor to apoptosis.

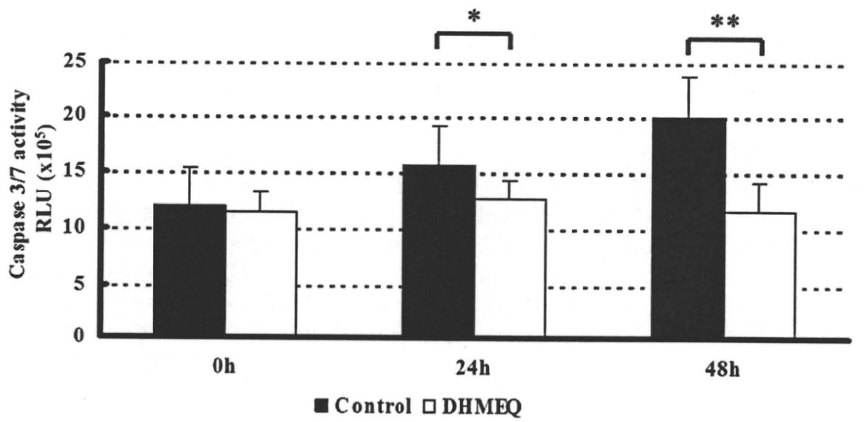

FIG. 6. Caspase 3/7 activity after isolation. Caspase 3/7 activity in the DHMEQ-treated group at 24 and 48 h after isolation was significantly lower than in the control group at the respective times. Results are representative of six independent experiments, and are expressed as means ± SD ($n = 6$). $^{*}P < 0.05$ *versus* control, $^{**}P < 0.01$ *versus* control.

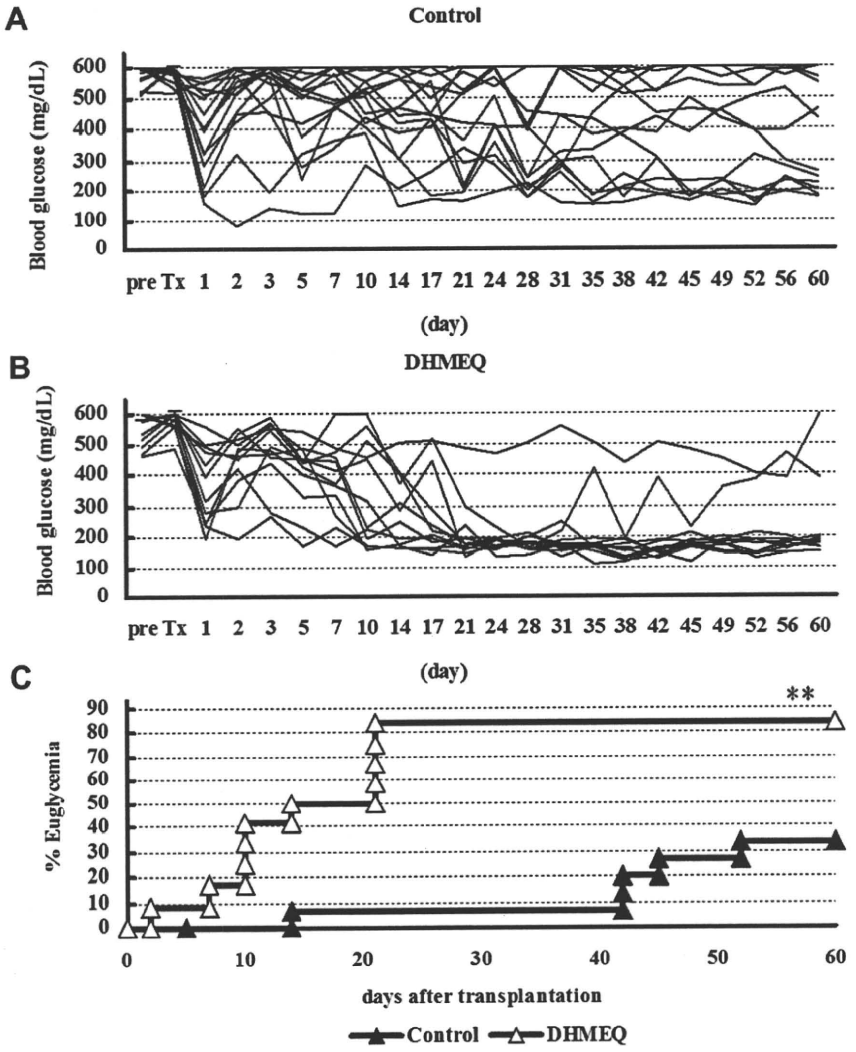

FIG. 7. Reversal of diabetes in STZ-induced diabetic mice by intraportal islet transplantation. (A) Control ($n = 16$). (B) DHMEQ ($n = 12$). (C) The rate of euglycemia of STZ-induced diabetic mice and days required to reverse diabetes (<200 mg/dL of blood glucose). The rate of euglycemia in the DHMEQ-treated group was significantly higher than that of the control. $^{**}P < 0.01$ *versus* control.

NF-κB consists mainly of a heterodimer from p65 (Rel A) and p50 proteins [7] and is constitutively expressed in the cytoplasm in an inactive form associated with an inhibitory protein called IκBα [23]. Exposure to cytokines such as TNFα and IL-1β allows liberated NF-κB to translocate into the nucleus. The activated NF-κB then regulates multiple proinflammatory genes including cytokines (IL-1, IL-6, TNFα, and IFNγ), MCP-1, and iNOS [7]. In islets, many studies have reported that exogenous cytokines activate intraislet NF-κB [24, 25] and that NF-κB regulates proinflammatory genes participating in islet dysfunction or insulitis [26]. Two reports have shown that NF-κB was activated during culture [21, 22]. So, we investigated whether the isolation procedure itself could activate NF-κB and whether DHMEQ, an NF-κB inhibitor, could inhibit its activation. We could not detect that NF-κB was activated during culture, but NF-κB was found to be activated immediately after isolation. As shown in Fig. 1, NF-κB activation levels in the cytosol are nearly the same between the control and DHMEQ-treated groups. This means that activated NF-κB was only detected in the nucleus, and its activation was effectively inhibited by DHMEQ.

To consider DHMEQ's effect on islet function, we performed GSIS. GSIS was the same between the groups (Fig. 2) and we could not detect the negative effect on islet function by DHMEQ. However, GSIS decreased rapidly from 12 h to 24 h in the control group. We considered that apoptosis in islets would be promoted during culture and that the secretory ability of islets would be declined. However, GSIS in the DHMEQ-treated

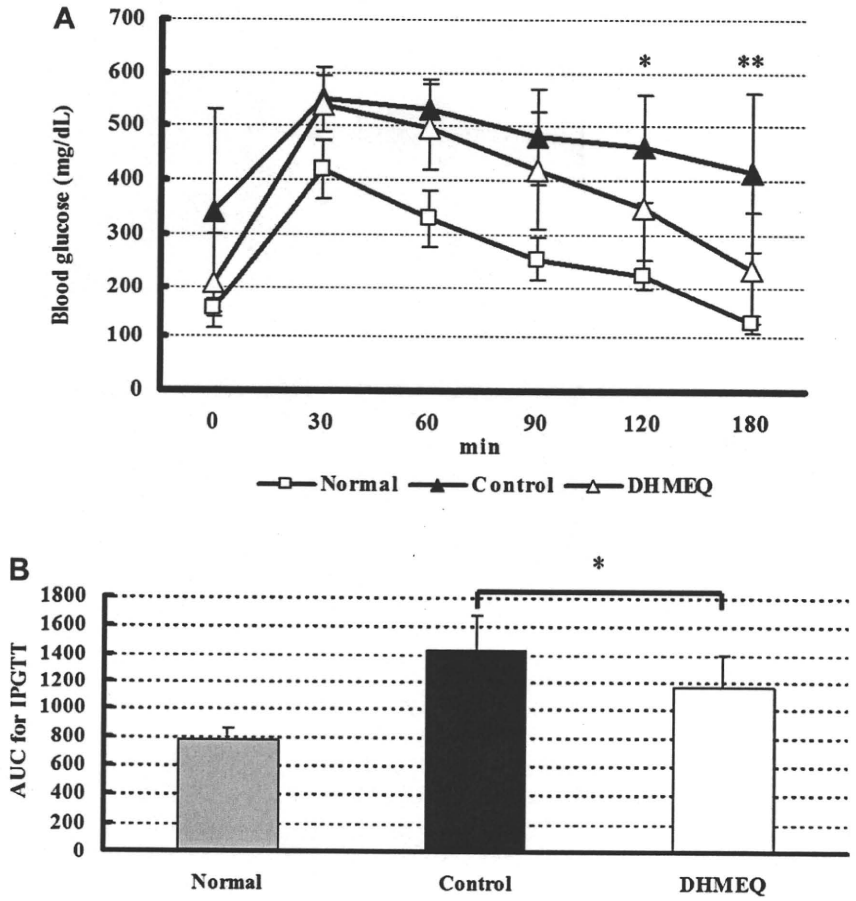

FIG. 8. IPGTT. (A) The data are representative of 12 independent experiments and are expressed as means ± SD (normal: $n = 8$, control: $n = 12$, DHMEQ: $n = 12$). The blood glucose levels of the DHMEQ-treated group were significantly lower than those of the control at 120 and 180 min. *$P < 0.05$ *versus* control, **$P < 0.01$ *versus* control. (B) The AUCs for IPGTT in the DHMEQ-treated group were significantly lower than those of the control. The data are expressed as means ± SD ($n = 12$). *$P < 0.05$ *versus* control.

group showed a smaller reduction than occurred in the control. This might be due to the possible preservation of the secretory ability of DHMEQ-treated islets by inhibiting islet apoptosis or the primary nonfunction might exist in the control islets.

The pharmacologic inhibition of NF-κB before transplantation may be an ideal therapeutic strategy to prevent β-cell death, to improve islet function and transplantation by a reduced amount of islets with a more viable β-cell mass. Some kinds of NF-κB blockade by protease inhibitor [24], IκBα superrepressor [12, 13], peptide-mediated transduction of the IκB kinase (IKK) inhibitor [6], and A20 overexpression [5] prevent IL-1β, IFNγ, and TNFα-induced β-cell dysfunction and death. These and our results suggest that DHMEQ is an ideal drug for protecting apoptosis and is superior to other NF-κB inhibitors in that it inhibits only the translocation of NF-κB, especially p65 without gene manipulation [15], and no adverse effects have been observed by *in vitro* and *in vivo* models. But DHMEQ can abrogate constitutive NF-κB activity and induce apoptosis in various cancer cell lines, such as hormone-refractory prostate cancer cells [27] and adult T-cell leukemia cells [28]. The mechanism to determine whether a cell survives or enters apoptosis by NF-κB activation depends on the cell type, the species, and the conditions to be stimulated. Other groups have reported that NF-κB protects β-cells from TNFα-mediated apoptosis and that NF-κB inhibition induces apoptosis in primary or insulin-secreting cells [29] or

TABLE 2

Insulin Contents of Islet-Transplanted Graft Liver

Group	Insulin contents (ng / liver)
Control group	1,194.49 ± 1,206.93 ($n = 13$)
DHMEQ-treated group	2,485.05 ± 1,481.06 * ($n = 11$)

The data are expressed as means ± SD.
*$P < 0.05$ *versus* control.