Background: Recent studies have suggested that epidermal barrier dysfunction contributes to the development of atopic dermatitis (AD) and other allergic diseases.
Objective: We performed a prospective, randomized controlled trial to investigate whether protecting the skin barrier with a moisturizer during the neonatal period prevents development of AD and allergic sensitization.
Methods: An emulsion-type moisturizer was applied daily during the first 32 weeks of life to 59 of 118 neonates at high risk for AD (based on having a parent or sibling with AD) who were enrolled in this study. The onset of AD (eczematous symptoms lasting >4 weeks) and eczema (lasting >2 weeks) was assessed by a dermatology specialist on the basis of the modified Hanifin and Rajka criteria. The primary outcome was the cumulative incidence of AD plus eczema (AD/eczema) at week 32 of life. A secondary outcome, allergic sensitization, was evaluated based on serum levels of allergen-specific IgE determined by using a high-sensitivity allergen microarray of diamond-like carbon–coated chips.
Results: Approximately 32% fewer neonates who received the moisturizer had AD/eczema by week 32 than control subjects ($P = .012$, log-rank test). We did not show a statistically significant effect of emollient on allergic sensitization based on the level of IgE antibody against egg white at 0.34 kU_A/L CAP-FEIA equivalents. However, the sensitization rate was significantly higher in infants who had AD/eczema than in those who did not (odds ratio, 2.86; 95% CI, 1.22-6.73).
Conclusion: Daily application of moisturizer during the first 32 weeks of life reduces the risk of AD/eczema in infants. Allergic sensitization during this time period is associated with the presence of eczematous skin but not with moisturizer use. (J Allergy Clin Immunol 2014;134:824-30.)

Key words: *Atopic dermatitis, atopy, allergic sensitization, food allergy, IgE, randomized controlled trial*

The prevalence of atopic dermatitis (AD) among children continues to increase, reaching 20% in some parts of the world; almost half of all children experience eczema within the first 2 years of life. AD reduces quality of life, such as by disturbing sleep, and should be considered a significant global burden of disease.[1-4]

Skin barrier dysfunction contributes to the development of AD, and dry skin often causes inflammation of eczematous skin. Filaggrin, a key component of the epidermal differentiation complex, is required for barrier function. Disruption of the gene encoding filaggrin *(FLG)* is associated with development of AD, as well as ichthyosis. Children with mutations in *FLG* have increased transepidermal water loss, even before AD develops.[2,5,6] The skin stratum corneum of infants is intact shortly after birth, but the water-sustaining barrier function of skin becomes adult like only after the first year of life.[7] Therefore it has been proposed that intensive emollient use in early life could prevent AD, especially in infants at high risk for AD (based on having a parent or sibling with AD). This hypothesis was investigated in a pilot study,[8] and a large-scale randomized controlled trial (RCT) is underway (Barrier Enhancement for Eczema Prevention trial; http://www.beepstudy.org/).[9] We initiated an RCT in 2010 to test the effects of an emulsion-type moisturizer (2e [Douhet] emulsion; Shiseido, Tokyo, Japan) in neonates at high risk for AD.

Several cohort studies have provided evidence that infants with eczema tend to have other allergic diseases, such as asthma, rhinitis, and food allergy.[10,11] Moreover, topical application of peanut oil to neonatal skin increased the infant's risk of peanut allergy, indicating epicutaneous sensitization to allergens.[12] Loss-of-function mutations in *FLG* are associated with a wide range of allergic diseases and sensitization to airborne and food antigens, even though filaggrin expression is limited to the skin and oral mucosa and has not been detected in the respiratory or intestinal mucosa.[6,13-15]

Primary prevention of allergic disease has been studied for many years. However, studies of avoidance of food allergens, aeroallergens, or both have generally produced disappointing results.[16] In this study we investigate whether daily application of moisturizer to neonates at high risk for AD prevents allergic sensitization, as well as development of AD. In addition to the outcomes of this RCT, we report that the presence of skin lesions (including AD) is a risk factor for allergic sensitization.

Abbreviations used
AD: Atopic dermatitis
DLC: Diamond-like carbon
FLG: Filaggrin gene
IRB: Institutional review board
NCCHD: National Center for Child Health and Development
OR: Odds ratio
RCT: Randomized controlled trial
UMIN-CTR: University Hospital Medical Information Network Clinical Trials Registry

METHODS

Trial design and participants

We performed an investigator-blinded, randomized, controlled, parallel-group study at the National Center for Child Health and Development (NCCHD) in Tokyo, Japan, from November 2010 through November 2013 (Fig 1). The NCCHD is the only national hospital for mothers and children in Japan, performing more than 1600 deliveries per year. After receiving approval from the institutional review board (IRB) of the NCCHD in August 2010, we invited expectant mothers with family histories of AD who visited the prenatal clinic of the NCCHD to participate in this trial. A high familial risk of AD was defined as a history of physician-diagnosed AD for at least 1 of the unborn baby's parents or siblings. Informed consent was obtained from the parents before delivery. After birth, the study doctors and a dermatology specialist confirmed the eligibility of each neonate on the basis of the inclusion criteria (eg, absence of treatment with corticosteroids) and exclusion criteria (eg, abnormal skin disorders, such as ichthyosis), which had been registered with the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR; UMIN000004544). The enrolled neonates were then randomly assigned to the intervention (n = 59) or control (n = 59) group (Fig 2).

The intervention group began receiving daily application of an emulsion-type emollient (2e [Douhet] emulsion) from the first week of life; petroleum jelly was prescribed to each infant in both groups on request by the IRB. Emollient was applied each day for 32 weeks. All infants were examined by the same blinded dermatologist from the NCCHD at scheduled visits and at weeks 4, 12, 24, and 32 of life. At each visit, the dermatologist examined the skin condition of the infant and recorded a diagnosis of AD, eczema, skin rash without pruritus, or healthy skin without any lesions. The worldwide and most validated criteria for diagnosis did not specify a time frame for AD development, describing a chronic or relapsing course,[17-19] and therefore it was not possible to diagnose an infant's AD immediately after his or her pruritic skin lesion emerged.

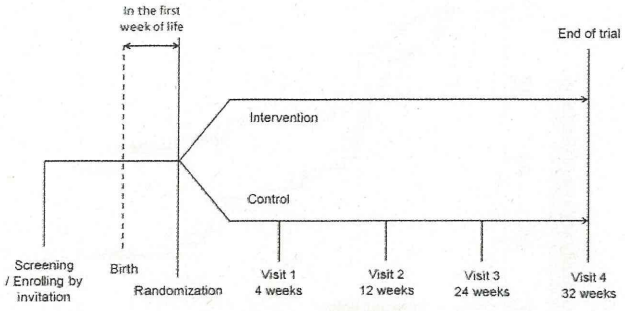

FIG 1. Study design.

Simpson et al[8] have modified the Hanifin-Rajka criteria for an incident case, setting the time for AD development to at least 2 weeks. The same authors proposed setting the time frame as at least 4 weeks.[20] We incorporated these criteria for an incident case of AD according to our definition of infantile eczema and AD. In our trial AD was defined as "itchy eczema at typical locations that lasted for at least 4 weeks," and infantile eczema was defined as the same eczema that lasted at least 2 weeks. Then these criteria were registered. Because AD and infantile eczema, as defined above, were essentially synonymous, we combined them as AD/eczema for this study. If an infant with skin rash or eczematous skin did not show any sign of pruritus, the dermatologist made a diagnosis of skin rash. When given a diagnosis of AD/eczema, infants were immediately removed from the study and treated appropriately. We instructed the parents to visit our outpatient clinic if their infants had any skin problems (Fig 1).

Outcomes

We registered this trial design, including the hypothesis and outcome measures, at UMIN-CTR (UMIN000004544). We proposed that protection of the skin barrier with a moisturizer beginning in the neonatal period would be a safe and effective strategy for prevention of AD and allergic sensitization. The primary outcome measure was the cumulative rate of incidence of AD, eczema, or both by temporal observation. The diagnostic criteria for infantile eczema, AD, or both (AD/eczema) were developed based on a modification of the United Kingdom Working Party's criteria and were applied by a dermatology specialist, as described above. Briefly, those criteria were a pruritic skin condition of at least 2 weeks' duration, visible flexural dermatitis (and/or on the cheeks and extensor surfaces), a history of dry skin, and a family history in a first-degree relative of the enrolled neonate.

Secondary outcome measures were the presence of allergen-specific IgE, transepidermal water loss (to measure stratum corneum hydration and pH at birth [baseline] and at weeks 4, 12, 24 and 32 of life; Vapo Meter, SW-4002; Delfin Technologies, Kuopio, Finland), stratum corneum hydration (Moisture Meter, SC-5; Delfin Technologies), stratum corneum pH (epidermal; Skin-pH-Meter, PH905; Courage & Khazaka Electronic GmbH, Koln, Germany), and skin colonization by *Staphylococcus aureus* (measured at the cheek).

Onset of allergic diseases, such as food allergy (registered on November 10, 2010), and onset of asthma were added as outcome measures on April 12, 2011, in response to a recommendation by the evaluation committee of the Ministry of Health, Labour and Welfare. Skin barrier functions were assessed by using the previously described methods.[21]

Statistical analyses

Analyses of the primary and secondary outcomes were conducted according to the intent-to-treat principle and based on the full analysis set, which included all randomized subjects. For an analysis of allergic sensitization, subjects without serum specific IgE (detected by using the diamond-like carbon [DLC] chip with high-density allergen immobilization and high sensitivity[22] at week 32; n = 2 for the intervention group and n = 5 for the control group) were excluded.

The primary outcome (cumulative rate of incidence of AD, eczema, or both by temporal observation) was analyzed by using the log-rank test. The

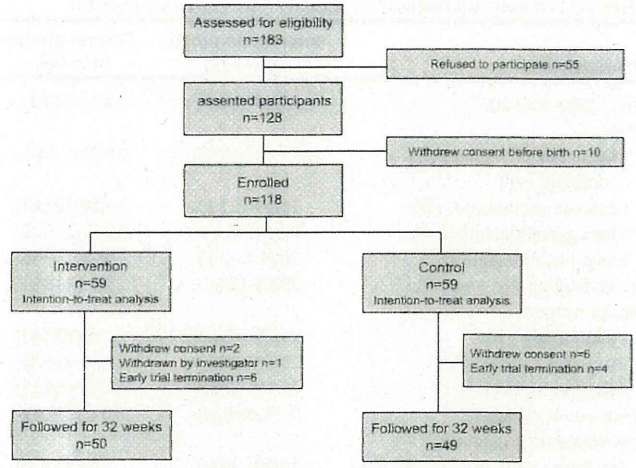

FIG 2. Study flow chart.

significance level was set at .05. The Kaplan-Meier method was used to estimate the cumulative incidence of AD/eczema for each group, and the Cox regression model was applied to estimate the hazard ratio between groups. The Mann-Whitney *U* test and χ^2 test with the Yates correction were used with continuous and categorical variables, respectively, to analyze secondary outcomes. Demographic and baseline data are presented as means, SDs, and proportions, as appropriate.

Once the data were collected from all subjects, we conducted several *post hoc* analyses. To evaluate the association between sensitization to foods and AD, we constructed a contingency table that dichotomized serum levels of antigen-specific IgE (based on results from the DLC assay) measured at week 32 at several cutoff values. The odds ratio (OR) and 95% CI were used to evaluate the degree of association. Statistical analyses were conducted with SPSS 17.0 software for Windows (SPSS, Chicago, Ill) and R software (version 3.0.1, http://www.R-project.org).

Consolidated Standards of Reporting Trials topics

Methods relating to Consolidated Standards of Reporting Trials statement (http://www.consort-statement.org/) and other methods are described in the Methods section in this article's Online Repository at www.jacionline.org.

RESULTS

Characteristics of neonates

We invited 183 expectant mothers from families at high risk for AD to participate in the study; 118 neonates were enrolled and randomly assigned to 2 groups of 59 infants each (Fig 2). Two infants assigned to the control group were found to have accidently received and used the emollient after opening the blinded data; 1 withdrew consent, and another completed the study without skin lesions. All 118 neonates were included as the intent-to-treat population (Table I) and the 2 infants who mistakenly received the intervention were classified into the control group. During the trial, 8 families withdrew informed consent (2 infants in the intervention group and 6 infants in the control group). The dermatologist withdrew an infant in the intervention group from the study because she or he had a hemangioma. After the second scheduled examination, we found that the incidence of AD was significantly lower in the intervention group than in the control group and reported this observation to the IRB of the NCCHD. The trial was discontinued at the recommendation of the NCCHD's IRB on November 30, 2013; by this time, 10 neonates had left the study (6 in the intervention group and 4 in the control group).

TABLE I. Baseline characteristics of the study population

Characteristic	Intervention group (n = 59)	Control group (n = 59)
Infant girl, no. (%)	26/59 (44.1)	24/59 (40.7)
Birth		
Mean ages of mothers at delivery (y)	35.8 ± 4.80	35.0 ± 4.85
Cesarean section, no. (%)	16/59 (27.1)	13/59 (22.0)
Mean gestational age (wk)	39.1 ± 0.97	39.0 ± 1.07
Mean birth weight (g)	3074 ± 363	3034 ± 366
Breast-feeding at 1 mo (%)	29/58 (50.0)	28/58 (48.3)
Family history		
Food allergy (%)	24/59 (40.7)	21/59 (36.8)
Bronchial asthma (%)	24/59 (40.7)	21/59 (36.8)
Allergic rhinitis (%)	46/59 (78.0)	48/59 (84.2)
Mean no. of siblings	0.34 ± 0.58	0.38 ± 0.62
Environmental exposures		
Smoking in the family, no. (%)	10/59 (16.9)	7/57 (12.3)
Any pet, no. (%)	12/58 (21.4)	13/57 (23.2)
Dog, no. (%)	8/58 (13.8)	6/57 (10.5)
Cat, no. (%)	2/58 (3.4)	4/57 (7.0)
Skin barrier function		
TEWL		
Mean lower leg	8.31 ± 2.67	8.40 ± 2.92
Mean forehead	8.29 ± 4.77	7.62 ± 3.15
Stratum corneum hydration		
Mean lower leg	13.7 ± 5.93	13.5 ± 5.94
Mean forehead	20.6 ± 10.7	19.2 ± 11.6
Mean pH	5.65 ± 0.59	5.61 ± 0.39

TEWL, Transepidermal water loss.

Among 118 infants evaluated, 47 had AD/eczema (19/59 in the intervention group and 28/59 in the control group), 13 had skin rash without pruritus (6 in the intervention group and 7 in the control group), and 31 did not have any skin lesions (20 in the intervention group and 11 in the control group). There were 5 infants (2 in the intervention group and 3 in the control group) who used moisturizers for skin disorders other than AD/eczema. The dermatology specialist stopped giving the emollient to 3 infants whose skin lesions seemed to be the result of urticaria or contact dermatitis caused by emulsion-type emollients (related adverse events). After several days, however, the doctor judged that these skin lesions were not adverse events because they disappeared rapidly and similar lesions were not seen when the same emollients were used again. These 3 infants did not have AD/eczema or skin rash when they were followed for 32 weeks. Among 8 families who withdrew consent, 2 families in the intervention group said that it was difficult for them to visit the NCCHD. There were no infants from families that withdrew consent who had skin lesions. In summary, adverse events caused by this emulsion-type emollient were not observed during this RCT.

Because the IRB recommended permitting application of petroleum jelly when the parents thought it necessary, we calculated the amount of these 2 types of moisturizers used by each group based on their diaries. The mean daily amount of emulsion-type moisturizer used by the intervention group was 7.86 ± 4.34 g (0 g for the control group, excluding the 2 infants placed in the wrong group). The mean daily amount of petroleum jelly applied to the control group was 0.101 ± 0.286 g (mean frequency of use, 0.235 d/wk). Petroleum jelly (20 g per bottle) was prescribed to all neonates born at the NCCHD, but we had no information about how much was used by the intervention group. Nevertheless, only a few of the parents occasionally used a small, almost ignorable, amount of the jelly on their infants.

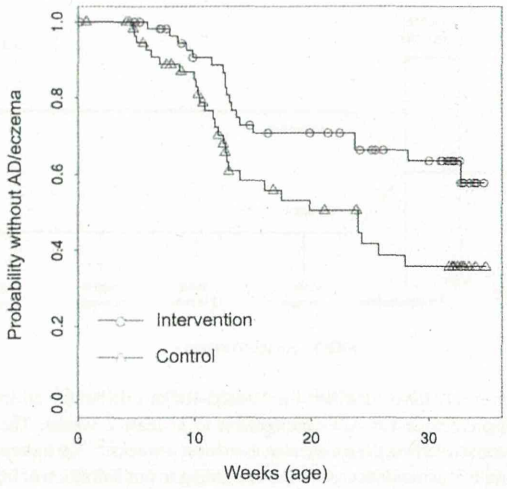

FIG 3. Proportions of infants who did not have AD/eczema. Kaplan-Meier plots show the proportions of infants in the intervention (*circle*) and control (*triangle*) groups with AD/eczema during the first 32 weeks of life. The log-rank test indicated statistically significant differences between groups ($P = .012$).

Primary and secondary outcomes

During their first 32 weeks of life, 19 infants in the intervention group had AD/eczema compared with 28 infants in the control group. Calculation of cumulative incidence values for AD/eczema by using the Kaplan-Meier method showed that the intervention group maintained intact skin for a significantly longer period than the control group ($P = .012$, log-rank test; Fig 3). Cox regression analysis showed the risk of AD/eczema to be significantly lower in the intervention group (hazard ratio, 0.48; 95% CI, 0.27-0.86).

In analyses of secondary outcomes (levels of allergen-specific IgE), we evaluated the serum levels of anti–egg white and anti-ovomucoid IgE in infants at 32 weeks,[22] as described in the Methods section of this article's Online Repository. IgE antibody data were converted to CAP-FEIA data after confirming the correlation between the data sets (see Fig E1 in this article's Online Repository at www.jacionline.org). However, we were not able to demonstrate a statistically significant effect of emollient on the rate of allergic sensitization based on level of IgE antibody against egg white (0.34 kU_A/L CAP-FEIA equivalents); the proportions of infants who were sensitized by allergen were similar in the intervention and control groups (Table II[18] and see Fig E2 in this article's Online Repository at www.jacionline.org).

The intervention group had significantly higher levels of stratum corneum hydration in the lower leg at weeks 12 and 24 compared with those seen in the control group (see Fig E3 in this article's Online Repository at www.jacionline.org). In both groups 6.1% of infants (7/115 cases measured) had positive test results for *S aureus* in cheek samples at birth, and 22.4% had positive test results (19/85 cases measured) at week 12. There was no significant difference between percentages of infants with positive test results for S aureus in the intervention (26.0%

TABLE II. Allergic sensitization at week 32

Level of specific IgE	Intervention group (n = 48)	Control group (n = 44)	*P* value†
Egg white (kU_A/L*)			
≥0.35	42% (20/48)	45% (20/44)	.88
≥0.70	38% (18/48)	45% (20/44)	.57
Ovomucoid (kU_A/L*)			
≥0.35	19% (9/48)	6.8% (3/44)	.17
≥0.70	13% (6/48)	4.5% (2/44)	.33

*We converted the levels of specific IgE (binding unit of IgE *[BUe]*/mL) measured with a DLC chip into CAP-FEIA equivalents (kU_A/L) based on a previously described method.[22] Cutoff values for allergic sensitization were set at 0.35 or greater or 0.7 or greater.
†The χ^2 test was used to calculate the difference between the 2 study groups.

TABLE III. Numbers of Infants with AD/eczema and allergic sensitization at week 32

Level of specific IgE	With AD/eczema (n = 43)	Without AD/eczema (n = 49)	*P* value†
Egg white (kU_A/L*)			
≥0.35	56% (24/43)	33% (16/49)	.043
≥0.70	56% (24/43)	29% (14/49)	.015
Ovomucoid (kU_A/L*)			
≥0.35	19% (8/43)	8.2% (4/49)	.24
≥0.70	12% (5/43)	6.1% (3/49)	.57

*We converted the levels of specific IgE (binding unit of IgE *[BUe]*/mL) measured with a DLC chip into CAP-FEIA equivalents (kU_A/L) based on a previously described method.[22] Cutoff values for allergic sensitization were set at 0.35 or greater or 0.7 or greater.
†The χ^2 test was used to calculate the difference between the 2 study groups.

[13/50 cases]) and control (17.1% [6/35 cases]) groups at week 12 (χ^2 analysis).

Post hoc analysis

Recent epidemiologic studies raised the possibility of epicutaneous sensitization to food allergens,[23] whereas others reported that some allergic diseases can be treated by repeated epicutaneous exposure to allergens.[24] Therefore we proposed the hypothesis that allergic sensitization can occur through eczematous but not healthy skin.[23] In a *post hoc* analysis of our data, we compared allergic sensitization in infants with and without AD/eczema at 32 weeks. We found that a greater proportion of infants with AD/eczema had allergic sensitization based on the serum levels of anti–egg white IgE (cutoff level of 0.34 kU_A/L CAP-FEIA equivalents) than infants without AD/eczema (P = .043, Table III).[18] The OR for allergic sensitization in infants with AD/eczema was 2.86 (95% CI, 22-6.73; Table IV[22] and see Fig E4 in this article's Online Repository at www.jacionline.org).

Thirteen infants of a total population had skin rash without pruritus. Six of these 13 infants also had allergic sensitization, and therefore we investigated whether there was an association between allergic sensitization and the presence or absence of skin lesions. The OR for allergic sensitivity (cutoff level of 0.34 kU_A/L CAP-FEIA equivalents) in infants with skin lesions compared with that in infants without skin lesions was 3.73 (95% CI, 1.49-9.36; Table IV and see Fig E4, *B* in this article's Online Repository at www.jacionline.org).

We have shown in this and previous studies that measurements of IgE by using a DLC chip correlate with those determined by using CAP-FEIA (see Fig E1). To prove the accuracy of CAP-FEIA equivalents measured by using a DLC chip in allergic sensitization, we calculated ORs for allergic sensitization using 25 different cutoff levels, ranging from 0.1 to 8.0 kU_A/L (Table IV). We found that ORs for allergic sensitization were greater for infants with AD/eczema than those without AD/eczema and for infants with compared with those without skin lesions when cutoff values were set at 25 different levels.

TABLE IV. Allergic sensitization based on cutoff levels of IgE specific for egg white at week 32

Cutoff values for specific IgE for egg white			
DLC chip (BUe/mL)	CAP-FEIA (kU_A/L)*	Skin lesion (+) vs others, OR (95% CI)	AD/eczema (+) vs others, OR (95% CI)
54.0	0.10	3.01 (1.27-7.16)	2.34 (1.001-5.48)
67.4	0.13	3.38 (1.41-8.07)	2.54 (1.09-5.95)
72.9	0.14	3.79 (1.58-9.14)	2.76 (1.18-6.48)
82.7	0.16	3.49 (1.46-8.39)	3.00 (1.28-7.06)
92.3	0.17	3.23 (1.35-7.71)	3.27 (1.39-7.72)
124.3	0.23	2.99 (1.26-7.11)	2.95 (1.26-6.90)
126.1	0.23	2.77 (1.17-6.57)	2.66 (1.15-6.20)
151.6	0.28	2.57 (1.08-6.08)	2.41 (1.04-5.59)
167.8	0.31	2.90 (1.21-6.93)	2.63 (1.13-6.12)
170.6	0.32	3.29 (1.36-7.97)	2.88 (1.23-6.72)
170.8	0.32	3.05 (1.26-7.39)	2.61 (1.12-6.08)
173.2	0.32	2.84 (1.17-6.85)	2.38 (1.02-5.52)
182.2	0.34	3.24 (1.32-7.96)	2.61 (1.12-6.08)
361.7	0.66	3.73 (1.49-9.36)	2.86 (1.22-6.73)
364.4	0.67	4.35 (1.69-11.2)	3.16 (1.33-7.49)
412.5	1.21	4.04 (1.57-10.4)	2.88 (1.21-6.81)
474.8	2.18	3.76 (1.46-9.67)	2.62 (1.11-6.20)
540.3	3.20	3.50 (1.36-8.99)	2.90 (1.21-6.94)
607.0	3.93	4.13 (1.55-11.0)	3.23 (1.33-7.82)
754.2	5.28	3.84 (1.44-10.2)	2.94 (1.22-7.12)
801.1	5.71	3.57 (1.34-9.51)	2.68 (1.11-6.50)
824.6	5.92	4.31 (1.55-12.0)	3.00 (1.22-7.39)
843.4	6.09	4.00 (1.44-11.1)	3.39 (1.35-8.49)
1004.2	7.56	3.71 (1.33-10.4)	3.09 (1.23-7.74)
1049.7	7.98	3.44 (1.23-9.62)	2.81 (1.12-7.06)

BUe, Binding unit of IgE.
*The levels of specific IgE (BUe/mL) measured with a DLC chip were converted into CAP-FEIA equivalents (kU_A/L) by using a previously described method.[22]

We detected loss-of-function mutations in *FLG* in 6 of the 57 DNA samples from infants. We were not able to demonstrate whether development of AD/eczema correlates with the presence of mutations, probably because of the small sample size (data not shown).

DISCUSSION

In a prospective RCT we investigated whether protection of the skin barrier with an emollient during the first 32 weeks of life prevents AD/eczema development in infants. A previous uncontrolled pilot study investigated whether a moisturizer can prevent AD,[8] but to our knowledge, this is the first RCT to investigate this question.

This trial was performed at only the NCCHD, mainly because of its logistic support. We tested the effects of an emulsion-type moisturizer (2e [Douhet] emulsion) because it is widely used, including for infants, and its composition has been disclosed. Studies to investigate the effects of other moisturizers on other populations are needed to support our findings.

One limitation of our study involves the diagnosis of AD. Worldwide and most validated criteria for the diagnosis of AD did not define the time frame of signs and symptoms,[17-19] resulting in its inability in diagnosis for early onset of AD in infancy. For this trial, we made the diagnosis of AD/eczema based on modified criteria proposed by Simpson et al.[8,20]

Intensive use of a moisturizer was reported to increase hydration of the stratum corneum in neonatal skin[25]; we confirmed this observation in our study. Daily application of an emulsion-type moisturizer during the first weeks of life increased stratum corneum hydration at week 12 compared with that seen in infants who occasionally received the minimum amount of petroleum jelly (control subjects). We found no statistically significant differences between the intervention and control groups in detection of *S aureus* in cheek samples or *FLG* mutations. This lack of association could be a result of insufficient statistical power, and therefore further studies are needed.

Primary prevention of allergic sensitization

Several cohort studies revealed that early-onset eczema increases the risk for allergic diseases, such as asthma, allergic rhinitis, and food allergy.[10,11] The presence of AD was the main skin-related risk factor for food allergen sensitization in young infants.[26] We confirmed that levels of anti–egg white and anti-ovomucoid IgEs measured by using a DLC chip correlate with those from CAP-FEIA. IgE-mediated egg allergy is one of the most common forms of food allergy; IgE against egg white is often used as a marker of atopy in infants.[27,28] In our study we were not able to show the significant effect of emollient on the prevention of allergic sensitization based on the level of IgE antibody against egg white; similar proportions of infants were sensitized in the intervention and control groups. However, we showed that a higher proportion of infants with AD/eczema had allergic sensitization based on serum concentrations of anti–egg white IgE compared with infants without AD/eczema. Furthermore, we found infants with skin lesions to have a more than 3-fold greater risk for allergic sensitization than infants without skin lesions based on 20 of 25 different cutoff points (range, 0.1-8.0 kU_A/L CAP-FEIA equivalents). Collectively, these findings indicate that the presence of eczematous skin, rather than a lack of emollient use, induces or promotes sensitization to allergens, such as egg white, during the first 8 months of life.

The mechanisms of this process are unclear. Levels of tight junction proteins (eg, claudin-1) between epidermal cells are significantly decreased in patients with AD compared with those seen in nonatopic subjects.[29] Also, Langerhans cells were reported to elongate their dendrites, penetrate keratinocyte tight junctions, and take up antigens when the Langerhans cells were activated by means of tape stripping.[30] These results could provide information on how eczematous skin promotes allergen sensitization.

Future directions

Findings from our RCT support our hypothesis that daily application of a moisturizer would prevent development of AD/eczema during the first 32 weeks of life. Contrary to our hypothesis, however, allergic sensitization, as assessed on the basis of acquisition of anti–egg white IgE, was not affected by application of the emollient. Our *post hoc* analysis revealed that the incidence of allergic sensitization was significantly increased among infants with skin lesions, including those caused by AD/eczema, compared with that seen in infants without these lesions. However, studies of a larger number of subjects might find that moisturizer use reduces allergic sensitization by preventing development of AD/eczema. In this *post hoc* analysis skin rash that did not fulfill the present criteria for AD/eczema, such as a lack of pruritus, was proposed to contribute to allergen sensitization. Allergic sensitization sometimes precedes and predicts the development of eczema,[31] and we have described the presence of low-affinity IgE against food antigens in blood and cord blood samples from newborns.[32] Therefore further studies should examine whether sensitization might occur through the placenta or neonatal gastrointestinal tract. It will be interesting to examine the temporal sequence of allergic sensitization, especially of epicutaneous sensitization to food antigens, by separately measuring levels of low-affinity and ordinary IgEs against food antigens.

We thank Professor Emiko Noguchi for providing information regarding the primer design for *FLG* mutations. We also thank Ms Kazuko Hayase and Ms Akiko Maruta of the NCCHD for their excellent assistance.

Clinical implications: Daily application of emollient reduces the risk of AD/eczema by 32 weeks. We might be able to reduce the prevalence of allergic sensitization by preventing the development of AD/eczema.

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METHODS

Interventions, randomization, and blinding

The research pediatricians (K.H. and K.M.) at the Division of Allergy of the NCCHD enrolled participants who met our criteria. Randomization of neonates into 2 groups was performed by means of random permuted blocks of size 4 at the Clinical Research Center of the NCCHD. The effect of intervention was evaluated as the cumulative incidence of AD/eczema, as registered at the UMIN-CTR. Dermatologists in the Division of Dermatology of the NCCHD examined the infants at scheduled visits in an investigator-blinded manner. The list of randomization was kept at the Clinical Research Center of the NCCHD until the end of the study to maintain the blinded state of the investigators.

The emollient used was an emulsion-type moisturizer, 2e emulsion, which was purchased from Shiseido. It was selected because it is commercially available and in widespread use in Japan, including for patients with AD and infants, and its composition has been disclosed. It contains glycerin, xylitol, butylene glycol, behenyl alcohol, batyl alcohol, hydrogenated polydecene, dimethicone, squalane, pentaerythrityl tetraethylhexanoate, Simmondsia chinesis (JOJOBA) seed oil, PEG-60 glyceryl isostearate, PEG-5 glyceryl isostearate, carbomer, potassium hydroxide, sodium metaphosphate, phenoxyethanol, tocopherol, and water (see also http://2e.shiseido.co.jp/products/emulsion.html) but not preservatives or mineral oils. The moisturizer was applied at least once daily to the whole body surface of infants in the intervention group. The participating families in both groups were routinely given a 20-g bottle of petroleum jelly at birth. As recommended by the IRB, we permitted all the families to use the petroleum jelly when they believed it necessary. They recorded the amounts of emulsion-type moisturizer and petroleum used each day. The families also kept a daily diary regarding their infants' skin condition (rash, erythema, itch, or scratch) and the areas to which the moisturizers were applied. We instructed the parents/caregivers to use commercially available soap with mild cleansing potency for their baby's bathing. Parents were instructed to bath their babies at least once a day. These instructions were just based on local customs. Blood samples (200 μL) were collected from each infant at weeks 1 (birth), 12, and 32. Swab samples to determine skin colonization were collected at weeks 1, 4, 12, and 32. Physical condition and skin barrier functions, such as the stratum corneum water concentration, were also evaluated at weeks 1, 4, 12, and 32.

Sample size

The sample size was calculated based on the preliminary results of our unpublished cohort study at the NCCHD. In that study infants at 6 to 8 months of age had a 47% cumulative prevalence of eczema, which was based on a modification of the questionnaire described in the International Study of Asthma and Allergies in Childhood report.[E1] Our experience shows that the rate of eczema assessed by using the modified International Study of Asthma and Allergies in Childhood questionnaire is always considerably higher than actual diagnoses by dermatologists; on the other hand, our invited participants (families) had a high risk of AD. Because we have no other verification tools for estimation, we estimated that 47% of infants who received a moisturizer in this study and 20% of infants who did not receive a moisturizer would have eczema, with 80% power at the 5% significance level and assuming a dropout rate of 5%. It was estimated that 37 cases were needed in each group. We noted that the rate of eczema is fairly high among infants born in the NCCHD compared with those born in other regions in Japan, although the reason is unclear. One might speculate that a high socioeconomic status could affect the rate because the average income of expectant parents at the NCCHD was estimated to be twice that of expectant parents in other regions.[E2] In addition, the IRB of the NCCHD did not allow us to use "participants who do not use emollients"; we gave petroleum jelly to all the participating parents so that they could apply it when they thought their baby's skin was very dry. Thus we adopted an adaptive study design; that is, we decided to re-estimate the sample size based on the results of interim analyses. The IRB of the NCCHD approved our study design in August 2010. In November 2012, based on the scheduled plan, we had performed the first interim analysis when half of the estimated participants reached the end point. The sample size of each group was calculated as 108 cases based on the first interim analysis. However, 4 control group participants had withdrawn informed consent, whereas only 1 intervention group participant had done so. As a result, we decided to emphasize the importance of the control group to the RCT when explaining the study to potential participants.

The second interim analysis was performed in November 2013, as had been scheduled. We enrolled a total of 118 neonates (59 in each group) and found that the incidence of AD was significantly lower in the intervention group than in the control group. We reported this to the IRB of the NCCHD according to its due process. We decided to discontinue the study at the recommendation of the IRB of the NCCHD on November 30, 2013, at which time 10 neonates (6 in the intervention group and 4 in the control group) were continuing in the study.

Methods to measure allergen-specific IgE antibodies

As secondary outcome measures registered at the UMIN-CTR, serum levels of several allergen-specific IgE antibodies were measured by using a novel allergen microarray on a DLC-coated chip, a high-sensitivity detection method for allergen-specific antibodies, as previously described.[E3] We used mainly a DLC chip method to measure allergen-specific IgE antibodies because it requires less than 2 to 5 μL of blood, although we sometimes measured the same allergen-specific IgE antibodies using the ImmunoCAP solid-phase IgE assay (CAP-FEIA; Thermo Scientific, Uppsala, Sweden) when the blood sample volume was sufficient. The DLC chip, but not CAP-FEIA, can detect low-affinity IgE antibodies that are present in fetuses and neonates.[E4] IgE antibody levels measured with a DLC chip correlate well with those determined by using CAP-FEIA when adult samples are used, and we confirmed this correlation by using our own neonatal samples when we had a sufficient blood volume to test. We successfully measured 3 allergen-specific IgE antibodies (to egg white, ovomucoid, and milk) using both a DLC chip and CAP-FEIA methods (Fig E1). For anti-milk antibody, correlation between the values obtained by using the 2 methods was not sufficiently high, suggesting the presence of low-affinity IgE antibodies. As a consequence, levels of anti–egg white and anti-ovomucoid IgE antibodies measured with a DLC chip correlated significantly with those determined by using CAP-FEIA and were used in further analyses. We were not able to validate the correlation between IgE antibodies detected with the DLC chip and those detected with CAP-FEIA in our samples at 1 and 12 weeks.

FLG mutation analysis

The representative *FLG* mutation sites found in the Japanese population were detected by using the primer sets described below. The p.R501*, p.S2889*, and p.S3296* mutations were screened by using TaqMan analysis (Life Technologies, Thermo Fisher Scientific, Waltham, Mass), as described previously.[E5,E6] The following mutations were screened for by using TaqMan analysis with newly developed primers and probes. The c.3321delA mutation was screened with 2 primers (5′-TGATAGTGAGGGACATTCAGAGGA-3′ and 5′-TTCATGAGTGCTCACCTGGTAGAT-3′) and 2 probes (5′-VIC-ACCTCCCCCTGACCAG-MGB-3′ and 5′-FAM-ACCTCCCCCGACCAG-MGB-3′). The p.Q1701* mutation was screened with 2 primers (5′-AGCA GACAGCTCCACAGACT-3′ and 5′-CTGTGTGTCTGACTCTTCTGAG-3′) and 2 probes (5′-VIC-CAGACAAGATTCATCTGT-MGB-3′ and 5′-FAM-GCAGATAAGATTCATCTGT-MGB-3′). The p.S2554* mutation was screened with 2 primers (5′-GCAAGCAGACAAACTCGTAACGAT-3′ and 5′-CTGGCTAAAACTGGATCCCCA-3′) and 2 probes (5′-VIC-CCAGGGA CAATCAGA-MGB-3′ and 5′-FAM-CCAGGGACAATGAGA-MGB-3′). The p.K4022* mutation was screened by using TaqMan analysis with 2 newly developed probes (5′-VIC-CGTTTGGTAAAGATCATC-MGB-3′ and 5′-FAM-CGTTTGGTTAAGATCAT-MGB-3′) and 2 primers (5′-TGTT TTCAAGGAAAGATCTGATATCTG-3′ and 5′-ATATATCACTAGAATG GCCACATAAACC-3′).

Bacterial culture of *Staphylococcus aureus*

Bacteria on the swabs obtained from the cheeks of infants were inoculated onto No. 110 *Staphylococcus* species–selective agar plates (Nissui Pharmaceutical, Tokyo, Japan) and cultured. Each bacterial colony was examined regarding the expression of *femA* and *femB* genes to confirm the presence of *S aureus*.

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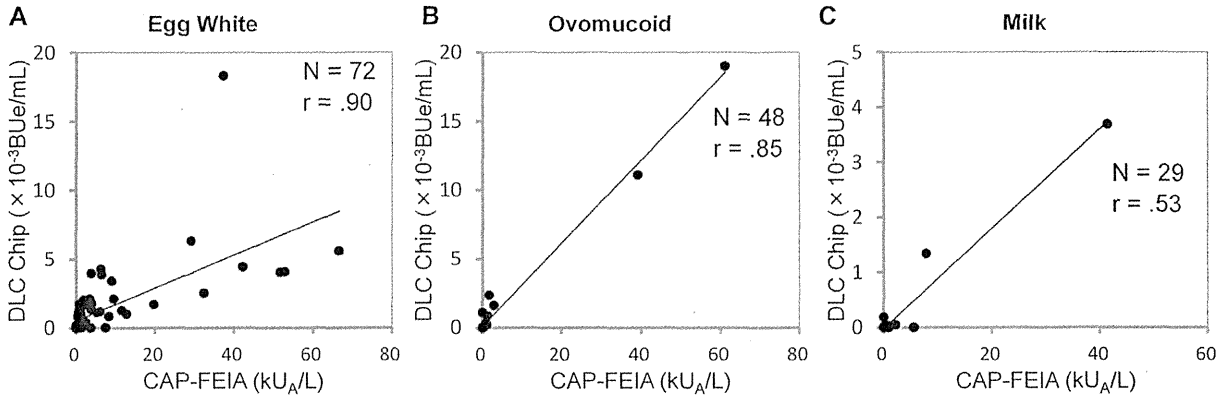

FIG E1. Correlation of allergen-specific IgE values determined by using a DLC chip system and CAP-FEIA. The values of anti-egg white **(A)**, anti-ovomucoid **(B)**, and anti-milk **(C)** IgE antibodies derived from 72, 48, and 29 infants, respectively, could be determined by using both the CAP-FEIA and DLC chip methods, and the correlations between these values obtained from the same samples were tested by means of linear regression analysis. *BUe*, Binding unit of IgE.

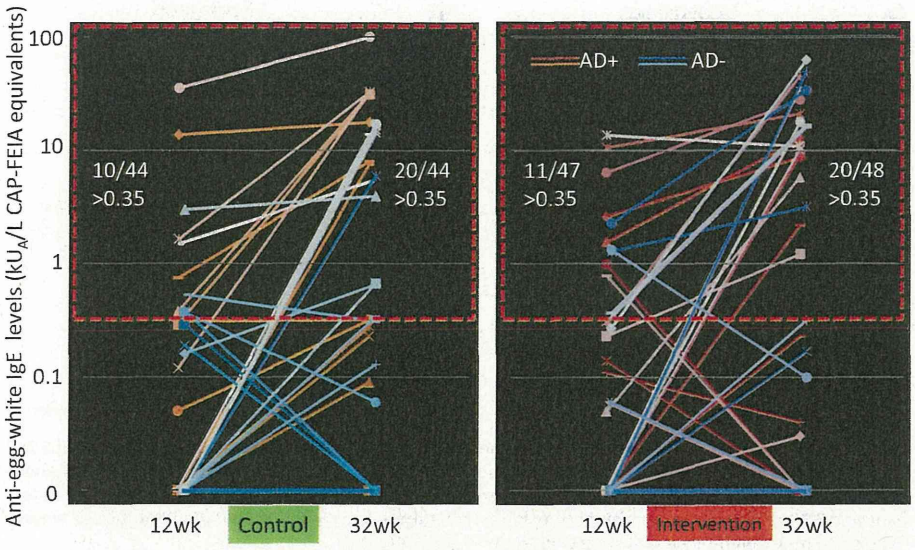

FIG E2. Allergic sensitization at weeks 12 and 32: comparison between the intervention and control groups. The serum levels of egg white–specific IgE (binding unit of IgE *[BUe]*/mL) in infants at weeks 12 and 32 were measured with a DLC chip and converted into CAP-FEIA equivalents (kU$_A$/L) by using a previously described method.[E3] Note that high correlation between these 2 data sets with the present samples was confirmed only at week 32. The values obtained from AD/eczema-positive infants are shown in warm colors, and those from AD/eczema-negative infants are shown in cold colors.

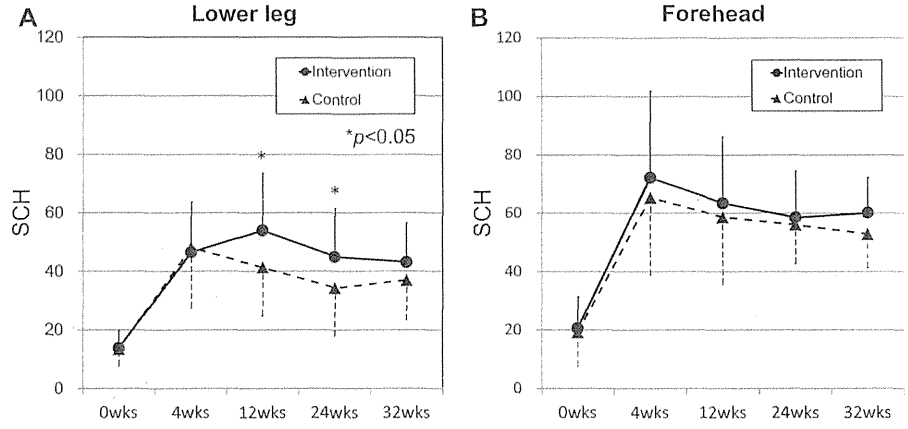

FIG E3. Stratum corneum hydration *(SCH)* change in the lower leg **(A)** and forehead **(B)** in each group. SCH values (relative impedance) on the outside of the lower leg (Fig E3, *A*) and forehead (Fig E3, *B*) were shown at baseline (week 0) and at 4, 12, 24, and 32 weeks of age. *Symbols* (*circles* and *triangles*) and *bars* stand for means and SDs. SCH values were significantly higher for the lower leg in the intervention group at 12 weeks of age compared with those in the control group ($P < .05$, ANOVA).

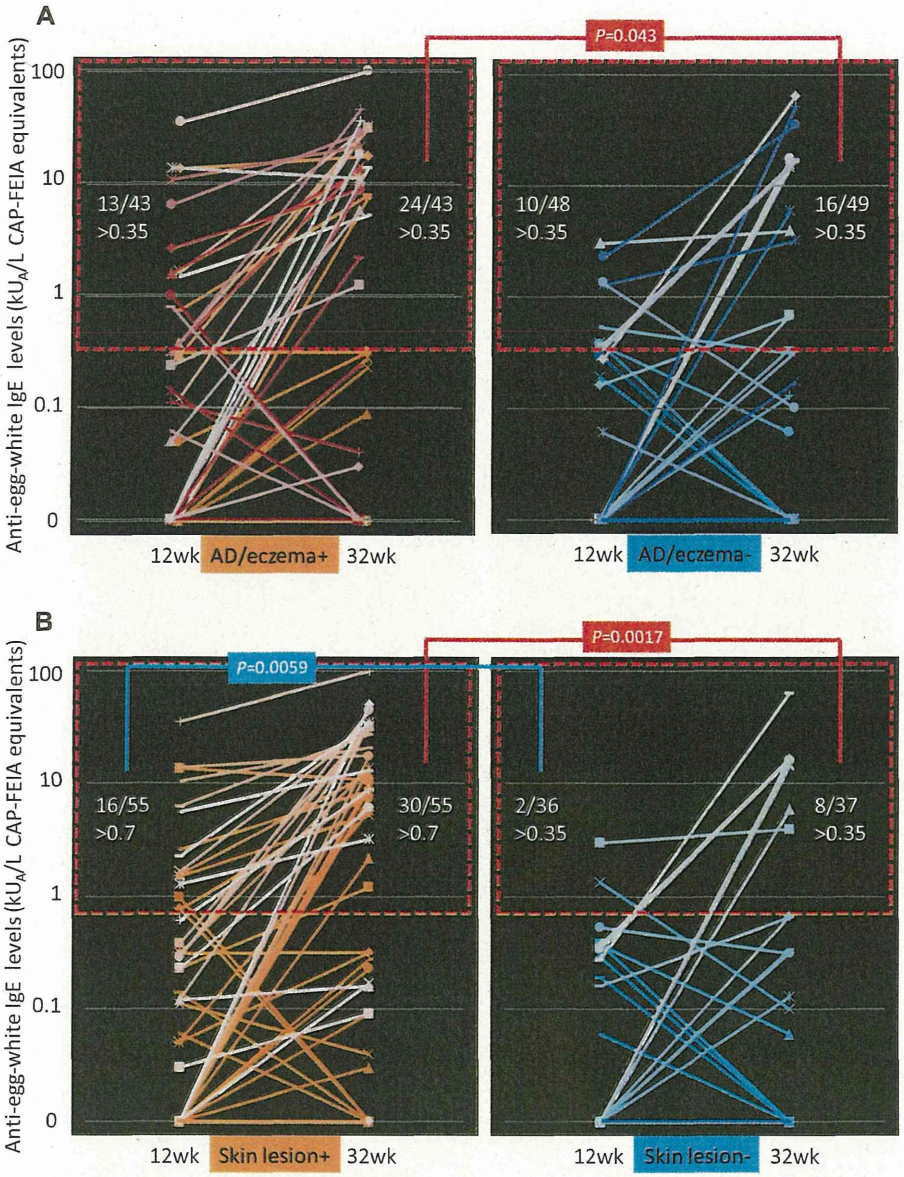

FIG E4. Allergic sensitization at weeks 12 and 32. Serum levels of egg white–specific IgE (binding unit of IgE *[BUe]*/mL) in infants at weeks 12 and 32 were measured with a DLC chip and converted into CAP-FEIA equivalents (kU_A/L). **A,** The AD/eczema-positive group had a higher proportion of infants sensitized with egg white at 0.35 kU_A/L CAP-FEIA equivalents at week 32 compared with the other group (P = .043). **B,** The skin lesion–positive group had a higher proportion of infants sensitized with egg white at 0.70 kU_A/L CAP-FEIA equivalent at week 12 (P = .0059) and week 32 (P = .0017) compared with the other group.

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Ibuprofen enhances the anticancer activity of cisplatin in lung cancer cells by inhibiting the heat shock protein 70

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Hsp70 is often overexpressed in cancer cells, and the selective cellular survival advantage that it confers may contribute to the process of tumour formation. Thus, the pharmacological manipulation of Hsp70 levels in cancer cells may be an effective means of preventing the progression of tumours. We found that the downregulation of Hsp70 by ibuprofen *in vitro* enhances the antitumoural activity of cisplatin in lung cancer. Ibuprofen prominently suppressed the expression of Hsp70 in A549 cells derived from lung adenocarcinoma and sensitized them to cisplatin in association with an increase in the mitochondrial apoptotic cascade, whereas ibuprofen alone did not induce cell death. The cisplatin-dependent events occurring up- and downstream of mitochondrial disruption were accelerated by treatment with ibuprofen. The increase in cisplatin-induced apoptosis caused by the depletion of Hsp70 by RNA interference is evidence that the increased apoptosis by ibuprofen is mediated by its effect on Hsp70. Our observations indicate that the suppression of Hsp70 by ibuprofen mediates the sensitivity to cisplatin by enhancing apoptosis at several stages of the mitochondrial cascade. Ibuprofen, therefore, is a potential therapeutic agent that might allow lowering the doses of cisplatin and limiting the many challenge associated with its toxicity and development of drug resistance.
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Subject Category: Cancer

The human Hsp70 family includes ≥ 8 highly homologous members that differ from each other by their intracellular localization and expression patterns.[1] Among them, the major stress-inducible Hsp70 (also called Hsp72) has an essential role in cell survival under stressful conditions. Compared with its normal counterpart, Hsp70 is often overexpressed in various cancer cells and is suspected to contribute to the development of tumours.[2,3] Indeed, the expression of Hsp70 in certain cancer types has been correlated with poor prognosis and resistance to chemotherapy.[4–6]

Tumour cells often express several proteins that, when abnormally elevated, render the tumour resistant to apoptosis.[7] Previous studies have confirmed not only that Hsp70 is cytoprotective, but also that it interferes effectively with cell death induced by a wide variety of stimuli, including several cancer-related stresses. Hsp70 is a potent inhibitor of the stress-activated kinase pathway, and apparently blocks apoptotic signals via interactions with JNK, Ask1 and SEK1.[8–11] Hsp70 is also a negative regulator of the mitochondrial pathway of apoptosis. Much of the focus on the antiapoptotic function of Hsp70 has been on events that occur after the disruption of the mitochondria. Hsp70 prevents the recruitment of procaspase-9 to the apoptosome, and its functional complex formation by direct interaction with apoptotic protease-activating factor 1 (Apaf-1).[12,13] Furthermore, Hsp70 inhibits the activation of caspase-3 and the cleavage of caspase-3 targets, such as ICAD and GATA-1.[14,15] On the other hand, recent studies have reported that Hsp70 can prevent apoptosis upstream of the mitochondria, by inhibiting events, which, ultimately, permeabilize the mitochondrial outer membrane, such as the activation of Bax.[16,17]

As a result of the inhibition by Hsp70 of the apoptosis induced by several anticancer drugs, as well as by other stimuli, we hypothesized that cancer cells would be sensitized to the induction of apoptosis by the neutralization of Hsp70. Hsp70 has been, indeed, targeted with pharmaceuticals, such as triptolide, quercetin and KNK437, which downregulate its expression.[18–20] Although they have prevented the progression of various cancer cells *in vitro* and *in vivo*,[21,22] the optimal clinical use of these small Hsp70 inhibitors singly or combined with other chemotherapeutics remains a challenge. Our overall objective was to pharmacologically control the levels of Hsp70, and increase the effectiveness of anticancer drugs.

Several experimental and epidemiologic studies and clinical trials have observed a powerful chemopreventive activity exerted by nonsteroidal anti-inflammatory drugs (NSAIDs).[23,24] The anti-carcinogenic properties of NSAID

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Keywords: Hsp70; apoptosis; ibuprofen
Abbreviations: Apaf-1, apoptotic protease-activating factor 1; COX, cyclooxygenase; HSF-1, heat shock factor 1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; NSAID, nonsteroidal anti-inflammatory drug; NSCLC, non-small cell lung cancer; PCR, polymerase chain reaction; RNAi, RNA interference; RT, reverse transcriptase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick and labelling
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have been attributed to their inhibition of cyclooxygenase (COX) enzymes. However, much higher doses of NSAID are needed to obtain an antitumoural effect than to inhibit COX,[25] suggesting that they also act via COX-independent mechanisms. On the other hand, NSAIDs, such as aspirin, salicylate and sulindac sulphide, inhibit the proliferation of cells and induce apoptosis in various cancer cell lines, which is considered an important component of their antitumoural activity, and increased sensitization of cancer cells to anticancer drugs.[26–29] There is currently interest in the ability of NSAID to directly lower the levels of antiapoptotic molecules, such as the Bcl-2 family[30] and 14-3-3 protein,[31] which inhibits the intrinsic mitochondria-dependent apoptosis in various cancer cells. Therefore, the NSAID-induced dysfunction of antiapoptotic proteins prompted us to examine whether other antiapoptotic molecules, including Hsp70, might also be targets in the prevention of tumour progression by NSAID. In this study, we show that ibuprofen is a potent inhibitor of Hsp70, which significantly suppresses its expression by depleting heat shock factor 1 (HSF1) in lung adenocarcinoma-derived A549 cells. The downregulation of Hsp70 by ibuprofen sensitized the cells to cisplatin, which was associated with the enhancement of cisplatin-induced apoptotic signalling. Ibuprofen did not only facilitate postmitochondrial events, including the activation of cisplatin-induced caspase-9, but also the activation of Bax, causing the release of cytochrome *c*. Besides the demonstration of a similar increase in the sensitivity of A549 cells to cisplatin conferred by Hsp70 knockdown and ibuprofen, these observations indicate that ibuprofen accelerates cisplatin-mediated apoptosis at multiple steps of the mitochondrial apoptotic pathway via the inhibition of Hsp70. We conclude that ibuprofen is a potential chemotherapeutic agent, which might enable (a) the use of lower, less toxic does of cisplatin and (b) the design of a new combination treatment of lung cancer.

Results

Ibuprofen suppresses the expression of Hsp70 in lung adenocarcinoma cells. To define the role of Hsp70 in promoting the formation of tumours, we first examined its expression in human lung cancer cell lines. Compared with BEAS-2B, a human, non-malignant, bronchial epithelial cell line, the expression levels of Hsp70 in lung cancer cells, such as A549 and H358 adenocarcinoma, were notably higher (Figure 1a). As in previous studies, which showed an increased expression of Hsp70 in various types of human cancers, including breast, pancreas and colon, we found that Hsp70 is also dysregulated in lung cancer cells. In this study, we screened conventional NSAID in search of a new pharmacologic inhibitor, which neutralizes Hsp70, as they induce apoptosis in cancer cells by selectively downregulating antiapoptotic proteins. The expression of Hsp70 after the exposure of A549 cells to various NSAID in non-toxic concentrations, was analyzed by immunoblot. Ibuprofen, in a 400-μM concentration, decreased the expression of Hsp70 by 23% in comparison with untreated cells, whereas other NSAID had no effect (Table 1). Figure 1b shows the decrease in Hsp70 protein and mRNA levels in A549 cells, after treatment with various concentrations of ibuprofen, *versus* no apparent decreases in Hsc70 and Actin. Ibuprofen also decreased the expression of Hsp70 in H358, a human lung adenocarcinoma cell line, in a dose-dependent manner (Figure 1c). These results suggest that ibuprofen decreases the expression of Hsp70 in various lung cancer cell lines.

Ibuprofen enhances the apoptosis induced by cisplatin by suppressing Hsp70. As ibuprofen prominently inhibited the expression of Hsp70, we next examined its effect on the proliferation of cancer cells. We observed no significant change in the viability of A549 and H358 cells after the

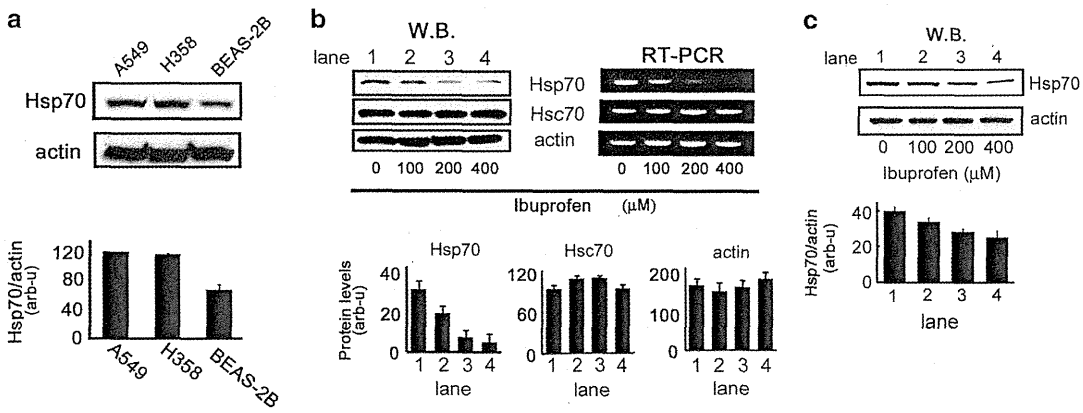


Figure 1 Ibuprofen suppresses the expression of Hsp70 in lung adenocarcinoma cells. (**a**) Upregulation of Hsp70 in lung cancer cell lines. Each cell extract was separated by SDS-PAGE and immunoblotted with an anti-Hsp70 or actin antibody (upper panel). The quantity of each protein was estimated by densitometric analysis using Scion Image (Scion, Frederick, MD, USA). The Hsp70/actin ratios are shown in the lower panel. (**b**) Effect of ibuprofen on the expression of Hsp70 protein and mRNA in A549 cells. Top: expression of Hsp70 and Hsc70 proteins in A549 cells treated with ibuprofen at the specified concentrations for 48 h, examined as described in **a** (left panel). Bottom: densitometric analysis of each protein level in arbitrary unit (arb-u). The alternation of each mRNA expression after ibuprofen treatment was analyzed by semiquantitative RT-PCR (top right panel). These results are representative of three separate experiments. (**c**) Effect of ibuprofen on the expression of Hsp70 protein in H358 cells. Expression of Hsp70 proteins in H358 cells treated with ibuprofen at the specified concentrations for 48 h (upper panel). The quantity of each protein was estimated by densitometric analysis (lower panel)

Table 1 Effects of nonsteroidal anti-inflammatory drugs on the expression of Hsp70 in A549 cells

NSAIDs	Hsp70 expression (%)
Ibuprofen (400 μM)	22.7 ± 2.8
Aspirin (2500 μM)	95.1 ± 7.8
Diclofenac (200 μM)	97.2 ± 5.6
Sulindac (15 μM)	98.9 ± 2.9
Piroxicam (60 μM)	96.6 ± 6.2
Indometacin (10 μM)	95.0 ± 15.1
Mefenamic acid (25 μM)	100.5 ± 6.0

Values are shown as means ± S.D.
The expression of Hsp70 was measured by immunoblotting with an anti-Hsp70 antibody. The quantity of Hsp70 protein was estimated by densitometric analysis using Scion Image. The values in parentheses are the highest non-toxic concentrations (approximately 90% viability) used for each NSAID on the A549 cells for 48 h

exposure to ~800 μM concentrations of ibuprofen alone, which downregulates Hsp70 (Figure 2a), while the exposure to 1.0 mM concentration of ibuprofen caused cell death. Combined, these observations indicate that the downregulation of stress-inducible Hsp70 was insufficient to cause the death of A549 and H358 cells.

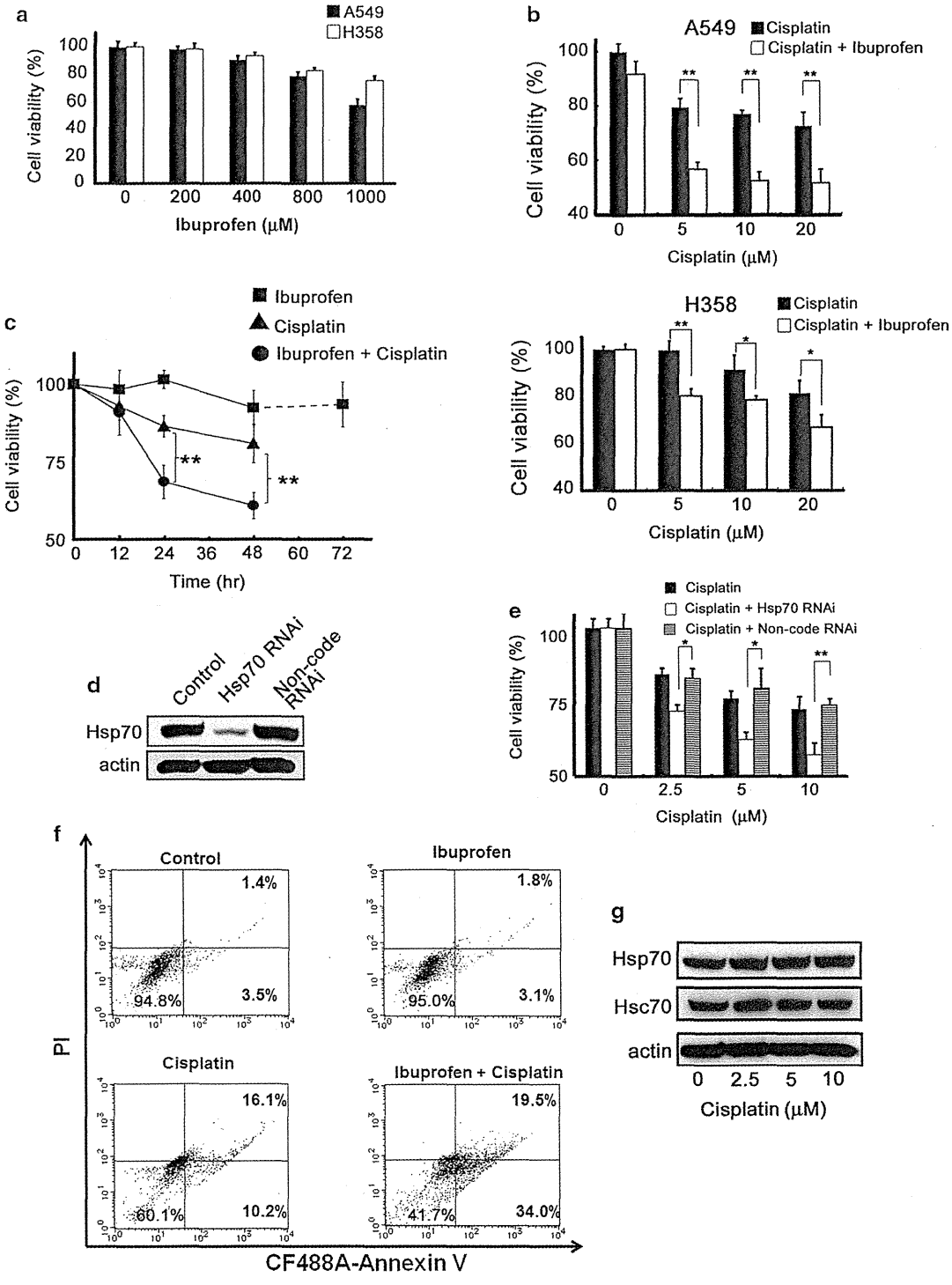
There is evidence that the inhibition of anti-apoptotic molecules, such as Hsp70, increases the sensitivity of tumour cells to anticancer drugs, thus improving the outcomes of chemotherapy. To study the therapeutic potential of ibuprofen, we examined whether its antitumoural effects are synergistic with those of cisplatin widely used in the treatment of lung adenocarcinoma. When we measured the survival of A549 (top of Figure 2b) and H358 (bottom of Figure 2b) cells exposed to increasing concentrations of cisplatin incubated in presence *versus* absence of ibuprofen, the latter prominently magnified the apoptosis induced by cisplatin, a synergistic effect confirmed by terminal deoxynucleotidyl transferase-mediated dUTP nick and labelling (TUNEL) staining (Figure 2c). To ascertain the effects conferred by the expression of Hsp70 on cell death, while excluding all effects of ibuprofen unrelated to Hsp70, we weakened the expression of Hsp70 by RNA interference (RNAi) (Figure 2d) and measured its effects on the apoptosis induced by cisplatin. The inhibition of Hsp70 decreased the viability of cisplatin-treated cells by approximately 20% (Figure 2e). Transfections with scrambled siRNA, serving as a control, showed no increase in cell death mediated by cisplatin. Cisplatin had no effect on the expression of Hsp70 (Figure 2g). We quantified the number of apoptotic cells in ibuprofen- and/or cisplatin-treated cultures using the CF488A-annexin V methods. Although cisplatin alone induced apoptosis in 10.2% of A549 cells, the co-treatment with ibuprofen increased the percentage of apoptotic cells to 34.0% (Figure 2f). These observations suggest that ibuprofen sensitizes A549 cells to cisplatin by decreasing the expression of Hsp70.

Ibuprofen decreases the expression of Hsp70 via transcriptional inactivation. The reverse transcriptase-polymerase chain reaction (RT-PCR) analysis described earlier revealed a decrease in RNA level following treatment with ibuprofen, suggesting that the expression of Hsp70 can be downregulated at the transcriptional level. After the recently discovered inhibition, by its antagonists, of the transcription of Hsp70 in cancer cells by blockade of the activation of HSF1[18,20] (which is often upregulated and constitutively activated in tumour formation), we studied the effects of ibuprofen on HSF1 in A549 cells. We first performed a ChIP assay to explore whether the inhibitory effect of ibuprofen is at the level of HSF1 DNA binding. As expected, we found an unequivocal association between HSF1 and the Hsp70 gene promoter containing the HSE site, in ibuprofen-untreated cells (Figure 3a). It is noteworthy that ibuprofen eliminated this binding (Figure 3a), suggesting that it inhibits the expression of Hsp70 via the action of HSF1. This also suggests that ibuprofen blocks the binding of HSF1 chromatin, or the steps which precede, in several processes needed to activate HSF1. Therefore, we broadened our analysis to examine the effect of ibuprofen on the expression of HSF1. Compared with unexposed, control cells, the HSF1 mRNA level was significantly lower in cells exposed to ibuprofen (bottom of Figure 3b). Consistent with its effect on the expression of mRNA, ibuprofen also decreased the expression of HSF1 protein in a dose-dependent fashion (top of Figure 3b). To confirm the inhibition of HSF1-mediated Hsp70 by ibuprofen, we lowered the amounts of HSF1 present in A549 cells by RNAi, and studied its effect on the expression of Hsp70. The treatment of cells with HSF1 dsRNA decreased the Hsp70 level compared with that measured in cells untreated with dsRNA (Figure 3c). Ibuprofen decreased the expression of HSF1 by 16% in comparison with untreated cells, whereas other NSAID had no effect (Table 2). Overall, these observations indicate that ibuprofen inhibited the expression of Hsp70 by depleting the HSF1 in A549 cells.

Ibuprofen accelerates the mitochondrial apoptotic process induced by cisplatin. Several studies have found that mitochondria might be a direct and important target of cisplatin in sensitive cells.[32,33] We studied the effects of ibuprofen on the depolarization of mitochondrial membranes and the cytochrome *c* release induced by cisplatin. A549 cells with or without cisplatin were incubated in absence or presence of ibuprofen and stained with JC-1. Treatment with cisplatin and ibuprofen lowered the mitochondrial membrane potential, manifest by an attenuated red and an enhanced green mitochondrial fluorescence (Figure 4a, lower right panel) compared with that observed with cisplatin alone (Figure 4a, upper right panel), while control (Figure 4a, upper left panel) or ibuprofen alone (Figure 4a, lower left panel) produced the red-dotted staining pattern of polarized mitochondria. The intensity of green mitochondrial fluorescence in cisplatin-treated cells is significantly increased (36.56 to 55.56%) by the co-treatment with ibuprofen. Ibuprofen also promoted the release of cytochrome *c* from the mitochondria induced by cisplatin (Figure 4b). These findings unequivocally indicated that, in A549 cells, ibuprofen enhanced the mitochondria-dependent apoptosis caused by cisplatin.

Ibuprofen increases the activation of Bax induced by cisplatin. The translocation of the pro-apoptotic protein Bax

to the mitochondria is closely associated with the apoptosis induced by cisplatin. To explore the mechanisms by which ibuprofen promotes the apoptosis mediated by mitochondria in response to cisplatin, we examined whether it was due to its ability to stimulate the translocation of Bax by cisplatin. We first monitored conformational changes in Bax as indicators of its activation. Western blot analysis of the immunoprecipitates with a conformation specific anti-Bax (6A7) antibody, which only recognizes the active form, revealed the presence of active Bax in A549 cells treated with cisplatin (Figure 5a, lane 4), although not in untreated cells (Figure 5a, lanes 1 and 2). Further exposure of the



cisplatin-treated cells to ibuprofen caused a 1.5-fold increase in active Bax, compared with incubation with cisplatin alone (Figure 5a, lane 3). When we analyzed the effects conferred by ibuprofen on the translocation of Bax to mitochondria in cisplatin-treated cells, we observed an approximately 1.3-fold increase in the amount of translocated Bax (Figure 5b). To exclude an effect of ibuprofen unrelated to the inhibition of Hsp70, we performed RNAi for a selective knock-down of Hsp70, and we studied its effects on the activation of Bax. Consistent with the earlier data presented for ibuprofen, the depletion of Hsp70 increased the activation of Bax in cisplatin-treated cells, although its extent was greater with Hsp70 RNAi than with ibuprofen (Figure 5c). These observations confirmed that (a) ibuprofen promotes the activation of Bax dependent on cisplatin and its translocation to the mitochondria in A549 cells and (b) its mechanism of action is mediated by the inhibition of Hsp70.

Ibuprofen facilitates events occurring upstream and downstream of mitochondrial disruption in cisplatin-mediated apoptosis. Previous studies have shown that Hsp70 can inhibit apoptosis by acting downstream of the mitochondria.[12–15] Hsp70 interacts directly with Apaf-1 to prevent the formation of cytochrome *c*-mediated apoptosome and subsequent activation of caspase-9. To examine whether ibuprofen also influences the downstream mitochondrial events, we measured its effects on the cleavage of procaspase-9 in the apoptosis mediated by cisplatin. With an anti-active caspase-9 antibody, fully processed caspase-9 was predominantly identified in cisplatin-treated A549 cells

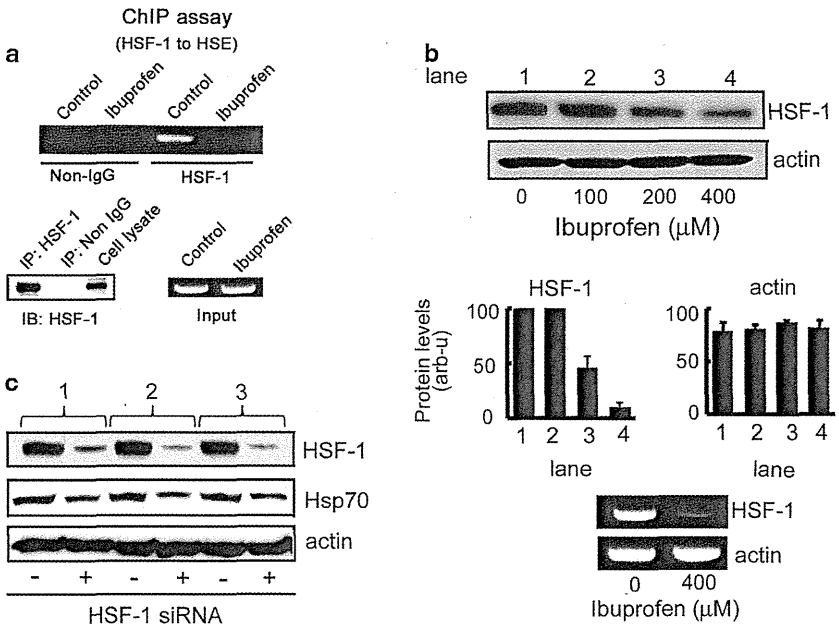


Figure 3 Ibuprofen inhibits the expression of Hsp70 by transcriptional inactivation. (**a**) ChIP assay for the association of HSF-1 with Hsp70 gene in A549 cells treated with or without ibuprofen. The DNA in the product immunoprecipitated by anti-HSF-1 or non-immune IgG was followed by PCR with a primer specific to the Hsp70 promoter. The immunoprecipitates with antibody against HSF-1 were confirmed by immunoblotting (bottom left). The actin signal is a control of DNA input (bottom right). (**b**) Effect of ibuprofen on the expression of HSF-1. Cell extracts from A549 cells with ibuprofen for 48 h were separated by SDS-PAGE and immunoblotted with a HSF-1-specific antibody (upper panel). The quantity of each protein was estimated by densitometric analysis (middle panel). The data are representative of three separate experiments. The effect of ibuprofen on the mRNA level of HSF-1 was confirmed by RT-PCR (lower panel). (**c**) HSF1-mediated inhibition of Hsp70 expression. A549 cells were treated with 10 nM siRNAs against HSF-1 or non-code siRNA. The HSF-1 silencing efficiency and its effect on the expression of Hsp70 were examined by immunoblotting, using appropriate antibodies. Three separate siRNAs oligo against HSF-1 were used for its knock-down. The results shown are representative of three separate experiments

Figure 2 Ibuprofen increased the antitumoural activity of cisplatin by suppressing Hsp70. (**a**) The viability of A549 (upper panel) and H358 (lower panel) cells treated with ibuprofen for 48 h was analyzed by MTT assay. The value is represented as the percentage of cell viability without ibuprofen, set at 100%. (**b**) Synergistic effect of ibuprofen on cisplatin-induced apoptosis in A549 (upper panel) or H358 (lower panel) cells. The cells were treated with the specified concentrations of cisplatin in absence or presence of 400 μM ibuprofen for 48 h, and the cell viability was assessed by MTT assay. The results are shown as means ± S.D. from triplicated experiments. The results shown are representative of three separate experiments. $^{*}P<0.05$; $^{**}P<0.01$ (by Student's *t*-test). (**c**) Time course of cisplatin-mediated cell death with ibuprofen. A549 cells were treated with 400 μM ibuprofen alone or A549 cells with 10 μM cisplatin were cultured in absence or presence of 400 μM ibuprofen, and the cell viability was analyzed by TUNEL staining. The results shown as means ± S.D. $^{**}P<0.01$ (by Student's *t*-test). (**d**) The silencing efficiency of Hsp70 determined by immunoblotting. (**e**) Effect of Hsp70 RNAi on the cisplatin-mediated death of A549 cells. The cells exposed to siRNA targeting Hsp70 or control siRNA were treated with 10 μM cisplatin for 48 h, and MTT assay was used to determine the cell viability. Data are presented as means ± S.D. from triplicated experiments. The results shown are representative of three separate experiments. $^{*}P<0.05$; $^{**}P<0.01$ (by Student's *t*-test). (**f**) Cytofluorimetric dot plot analysis of the CF488A-Annexin V *versus* propidium iodide (PI) staining performed in 10 μM cisplatin-treated or -untreated A549 cells in absence or presence of 400 μM ibuprofen for 48 h. A representative experiment out of three performed with similar results is shown. (**g**) Effect of cisplatin on the expression of Hsp70. The data are representative of three separate experiments

(Figure 6a, lane 3) over untreated cells (Figure 6a, lanes 1 and 2). It is noteworthy that treatment with ibuprofen increased >4-fold the amount of active caspase-9 in cells treated with cisplatin, compared with cells incubated with cisplatin alone (Figure 6a, lane 4). As, as reported earlier, the highest increases in the activation of Bax and release of cytochrome *c* by ibuprofen were <2-fold, these observations suggest that ibuprofen also facilitates the post mitochondrial process taking place between the release of cytochrome *c* and the activation of caspase-9. To verify that this is a specific effect, we studied the effect of Hsp70 knock-down on the activation of caspase-9 mediated by cisplatin. The caspase-9 activity in cells depleted of Hsp70 with cisplatin was fourfold greater than in control (scrambled) siRNA-treated cells (Figure 6b). We obtained similar results when we measured the activity of caspase-9 in cells treated with ibuprofen (Figure 6c) or siRNA against Hsp70 (Figure 6d) by a fluorometric assay using a synthetic substrate.

Table 2 Effects of nonsteroidal anti-inflammatory drugs on the expression of HSF-1 in A549 cells

NSAIDs	HSF-1 expression (%)
Ibuprofen (400 μM)	16.2 ± 3.9
Aspirin (2500 μM)	93.5 ± 2.9
Diclofenac (200 μM)	96.7 ± 6.6
Sulindac (15 μM)	99.8 ± 3.6
Piroxicam (60 μM)	96.3 ± 4.7
Indometacin (10 μM)	98.1 ± 1.6
Mefenamic acid (25 μM)	98.5 ± 1.1

Values are shown as means ± S.D.
The expression of HSF-1 was measured by immunoblotting with anti-HSF-1 antibody. The quantity of HSF-1 protein was estimated by densitometric analysis using Scion Image. The values in parentheses are the highest non-toxic concentrations (approximately 90% viability) used for each of the NSAID on the A549 cells for 48 h

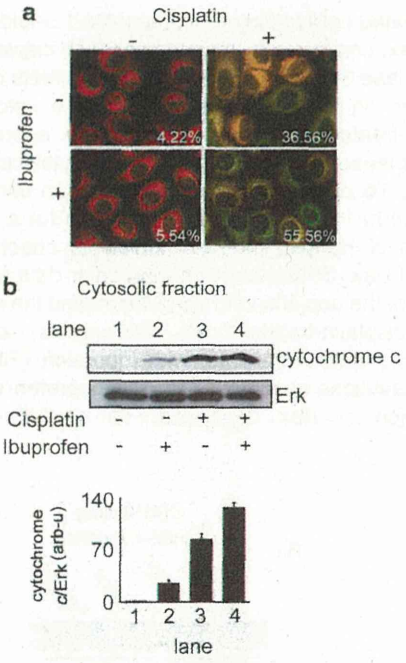


Figure 4 Ibuprofen exposure enhanced the cisplatin-dependent mitochondrial membrane depolarization and cytochrome *c* release. (**a**) A549 cells were treated for 48 h with 10 μM cisplatin, 400 μM ibuprofen, or both, and subjected to JC-1 staining to study the changes in mitochondrial membrane potential. The percentages indicate the green fluorescence intensity of JC-1 measuring with FACSCalibur. A representative experiment out of three performed with similar results is shown. (**b**) A549 cells, treated as described earlier, were fractionated into cytosol, and the release of cytochrome *c* was analyzed by western blot, using anti-cytochrome *c* antibody. The expression of Erk was monitored as an internal control of cytosol protein. The quantity of each protein was estimated by densitometric analysis. The results are means of three separate experiments from cells in different cultures

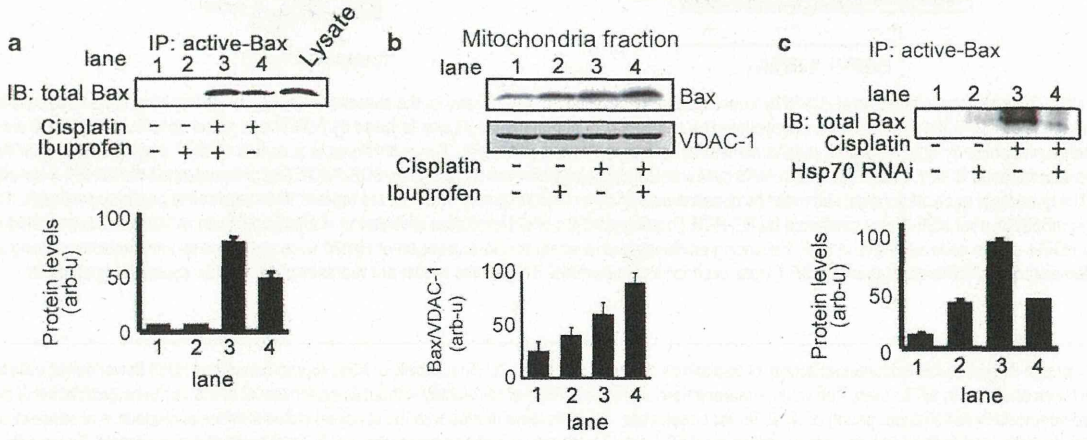


Figure 5 The downregulation of Hsp70 increased the cisplatin-mediated activation of Bax and its translocation to the mitochondria. (**a**) Detection of active Bax. A549 cells were treated with cisplatin (10 μM) and/or ibuprofen (400 μM) for 48 h. Active Bax was immunoprecipitated with an active conformation-specific monoclonal antibody and revealed by immunoblotting with an anti-Bax polyclonal antibody. The quantity of active Bax was estimated by densitometric analysis. (**b**) A549 cells, treated as described earlier, were lysed and fractionated by differential centrifugation to separate the mitochondria from the cytosol. The translocation of Bax to the mitochondria was visualized by the immunoblot of mitochondrial fractions, using an anti-Bax antibody. VDAC-1 was used as a loading control to ensure the use of equal amounts of mitochondria. (**c**) A decrease in Hsp70 by RNAi promoted cisplatin-dependent activation of Bax. A549 cells treated with Hsp70 or control siRNA were incubated in presence or absence of cisplatin; each cell extract was immunoprecipitated with an anti-active Bax antibody, followed by immunoblotting with anti-Bax antibody. The data are representative of three separate experiments

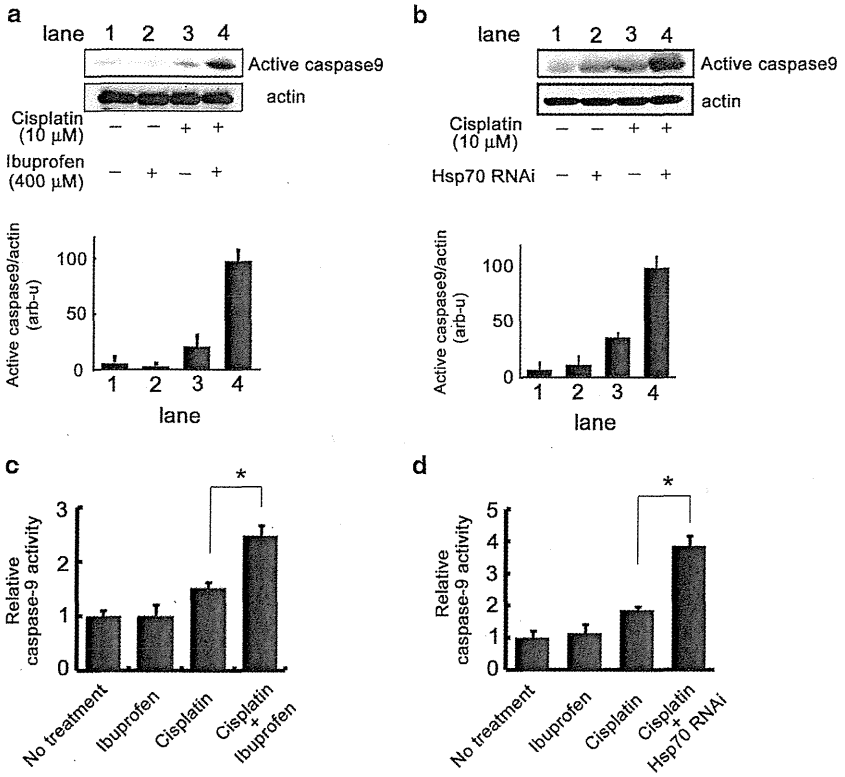


Figure 6 Synergistic effect of Hsp70 suppression on the cisplatin-mediated activation of caspase-9. (**a**) A549 cells were treated with cisplatin and/or ibuprofen, and cell extracts were immunoblotted with active caspase-9 antibody. The lower panel shows the measurement of each caspase-9. (**b**) A549 cells exposed to siRNA targeting Hsp70 or control siRNA were incubated with or without cisplatin, and the active caspase-9 was detected by western blot using an anti-caspase-9 antibody. The quantity of each protein was estimated by densitometric analysis (lower panels). (**c** and **d**). Assay for enzymatic activity of caspase-9, using a fluorogenic substrate. (**c**) After the incubation of the A549 cells with cisplatin (10 μM) and/or ibuprofen (400 μM), the caspase-9 activity of each cell extract was measured as described in Materials and methods section. (**d**) A549 cells transfected with Hsp70 siRNA or control siRNA were exposed to cisplatin for 48 h. The caspase-9 activity was then assessed, using an enzymatic assay as described earlier. The value of caspase-9 activity was presented relative to the activity in untreated cells, set at 1.0. The data represent mean values of three separate experiments. Significances were determined by Student's *t*-test (*$P<0.05$)

Overall, these observations confirmed unambiguously that ibuprofen intensified the apoptosis induced by cisplatin by its effects on the events occurring downstream of the mitochondria, by inhibiting Hsp70, although whether it stimulated the formation of apoptosome (essential for the recruitment of procaspase-9) remains to be determined. We conclude that ibuprofen promotes the apoptosis induced by cisplatin at multiple stages of the mitochondrial cascade, by attenuating the expression of Hsp70 in A549 cells.

Discussion

We found that, compared with non-malignant bronchial epithelial cells, human lung cancer cells overexpressed Hsp70. This is an important observation, as targeting the expression or function of Hsp70 has been suggested as an effective treatment strategy in several cancers, based on the hypothesis that higher levels of Hsp70 protect against cell death and increase the survival rate against modalities used in chemotherapy.[11,15] In fact, it is well documented that the expression of Hsp70 is significantly increased in cancer tissues and/or serums obtained from patients with non-small cell lung cancer (NSCLC)[34–38] and its overexpression correlates with poor prognosis in NSCLC.[36] Several reports have indicated that functionally related small molecules that inhibit Hsp70 decrease the viability of colo-rectal or pancreatic cancer cells by promoting apoptosis via the downregulation of Hsp70, and may be a promising new class of cancer chemotherapeutics.[19,21,22] We showed that ibuprofen, a relatively non-toxic and widely used NSAID, significantly decreased the expression of Hsp70 in lung adenocarcinoma cell lines. We also clearly demonstrated that the inhibitory mechanisms of ibuprofen on Hsp70 are due to a decrease in HSF1 expression. Although the fundamental mechanism behind the reduction in HSF-1 expression is unknown, a previous study has indicated that the nuclear factor 1 family member NFIX, which codes for site-specific DNA-binding proteins known to have multiple roles in replication, signal transduction and transcription, exerts a transcriptional repressive effect on the expression of HSF1 in cancer cells.[39] Whether NFIX is, indeed, involved in the inhibition of HSF1 expression evoked by ibuprofen is applicable in further studies. To the best of our knowledge, this is the first study of the inhibitory effects of NSAID on the cellular expression of Hsp70. In addition, we showed that ibuprofen does not influence the cell viability without additional stimuli, unlike its

maximal effect on the expression of Hsp70. The lack of inhibitory efficacy of ibuprofen against tumours is consistent with a previous study, which showed that low-dose ibuprofen did not induce apoptosis in mouse and human colorectal cancer cell lines.[29] Similar observations were made following RNAi of Hsp70, suggesting that the attenuation of Hsp70 *per se* is insufficient to cause the death of A549, and perhaps other cells. It has been shown that the knockdown of Hsp70 has no effect on the viability of several cancer cell lines, although sensitized them to anticancer drugs.[40,41] Therefore, the therapeutic potential of ibuprofen combined with chemotherapeutic agents needs to be explored.

Cisplatin is one of most effective chemotherapeutic drugs against NSCLCs.[42] It is noteworthy that damage to DNA caused by cisplatin enables apoptosis involving mitochondrial pathways, which is negatively regulated by Hsp70. As ibuprofen prominently suppressed the expression of Hsp70 in A549 and H358 cells, we examined the possible synergistic activity of ibuprofen and cisplatin against cancer. As expected, ibuprofen potentiated synergistically the anti-proliferative effect of cisplatin in A549 and H358 cells. Despite its potent antitumoural properties, the therapeutic use of cisplatin in oncology is seriously limited by dose-dependent adverse effects and frequent development of drug resistance.[43] Therefore, our findings may make useful contributions toward the development of new and less toxic chemotherapy against NSCLCs.

We also examined the molecular mechanisms of these synergistic properties of ibuprofen. Hsp70 protects cells against mitochondria-dependent apoptosis at different levels, although the precise mechanism remains hypothetical, because of regular contradictory descriptions of Hsp70 function. Earlier reports have shown a protective effect of Hsp70 against cellular apoptosis by inhibition of the apoptosome function, a protein complex comprising Apaf-1 and cytochrome *c*.[12,13] However, recent reports have questioned this repression of apoptosis downstream of the mitochondrial membrane permeabilization. Several studies have suggested that Hsp70 functions upstream of the mitochondria by preventing the release of cytochrome *c*, instead of inhibiting the apoptosome or other downstream points in the caspase cascade.[16,17] Some of this confusion may be due to different experimental systems used to evaluate apoptosis, or reflects the variability of apoptotic pathways among different cell lines. In this study, we found that the inhibition of Hsp70 by ibuprofen facilitates the activation of Bax induced by cisplatin, and its translocation to the mitochondria in A549 cells. This finding is consistent with the previous observation of blockade of Bax activation being one of the upstream sites of action of Hsp70. On the other hand, the role played by Hsp70 in the A549 cellular mitochondrial apoptotic pathway is likely to be more complex than described earlier because, in cisplatin-treated cells, the decreased expression of Hsp70 caused by ibuprofen amplified the activation of caspase-9 significantly compared with that of Bax. Furthermore, the similar increase in the activation of cisplatin-dependent Bax and release of cytochrome *c* by ibuprofen suggests that Hsp70 also inhibits the post mitochondrial steps between the release of cytochrome *c* and the activation of caspase-9. If Hsp70 were acting downstream of the mitochondria, one would predict that it interferes with the activation of caspase-9 in response to cytochrome *c*, either by inhibiting the formation of the apoptosome, or by preventing the binding of pro-caspase-9 to this complex. When we studied the effects of Hsp70 on the formation of, and recruitment of pro-caspase-9 to, the apoptosome, the cell lysates were immunoprecipitated, although Hsp70 failed to migrate with Apaf-1, cytochrome *c* or caspase-9 (data not shown). These results may be supported by previous report that no association between Hsp70 and Apaf-1 or apoptosome complex was observed, even under *in vitro* activation of caspase by the addition of cytochrome *c* and dATP.[44] Furthermore, we were unable to identify a new target for Hsp70 in the process of caspase-9 activation, indicating that its inhibitory activity is attributable to another indirect effect, instead of a direct one as previously reported. Altogether, the data presented here are the first evidence of cell death inhibition by Hsp70, by its targeting of both upstream and downstream mitochondrial processes, while the precise mechanisms by which it interferes with the activation of caspase-9 remains to be clarified.

In conclusion, ibuprofen potentiated the antitumoural properties of cisplatin in the cells of lung adenocarcinoma, via a mechanism of action mediated by the suppression of Hsp70. These findings may promote the development of a new strategy to increase the effectiveness of cisplatin in the treatment of NSCLCs, as well as highlight the putative merits of developing anticancer treatments targeting Hsp70.

Materials and Methods

Materials. The mouse monoclonal anti-Hsp70, the rat monoclonal anti-Hsc70 and rabbit polyclonal HSF-1 antibodies purchased from Stressgen – Enzo Life Sciences, Inc., Plymouth Meeting, PA, USA. Anti-Bax rabbit polyclonal (N-20) and anti-VDAC-1 goat polyclonal (N-18) antibodies were purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA. Anti-cytochrome *c* mouse monoclonal antibody (556433) was obtained from BD Pharmingen Inc., San Diego, CA, USA. Anti-caspase 9 and -ERK antibodies were acquired from Cell Signaling Technology Inc., Danvers, MA, USA. The mouse monoclonal antibody against actin was obtained from Chemicon International Inc., Temecula, CA, USA. Anti-Bax 6A7 monoclonal antibody and other reagents were purchased from Sigma-Aldrich, St. Louis, MO, USA.

Cell culture and viability assay. A549 and H358 lung cancer cell lines were cultured in Dulbecco's modified Eagle's medium containing 10% foetal bovine serum at 37 °C. BEAS-2B cells were grown in bronchial epithelial basal medium. All NSAID and cisplatin were dissolved in dimethyl sulphoxide and added to the medium at indicated concentrations. The activity of mitochondrial dehydrogenase 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to measure cell death/survival. The reaction product was measured at A_{570}, and the relative viability of cells treated with reagents *versus* untreated cells was calculated.

TUNEL staining. The TUNEL assay was performed using an *in situ* cell death detection kit (F Hoffmann-La Roche, Basel, Switzerland) according to the manufacturer's instructions. The ratio of TUNEL-positive cells to the total number of cells was calculated.

Immunoprecipitation and cell fractionation. A549 cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM sodium orthovanadate, 1 mM EDTA, 0.1% NP-40, 10 mM NaF) containing the Calbiochem Protease Inhibitor Cocktail Set III (Merck KGaA, Darmstadt, Germany). The cell lysates and immunoprecipitates were resolved in Laemmli sample buffer. The samples underwent sodium dodecyl sulphate-polyacrylamide gel electrophoresis were transferred to a polyvinylidene difluoride membrane, reacted with the respective antibodies, and detected with an ECL chemiluminescence detection kit (GE Healthcare, Fairfield, CT, USA). For the immunoprecipitation, the cell lysates were