developed $Abca12^{-/-}$ mice and the mice also confirmed the clinical features of HI [Zuo et al., 2008]. In addition, a mouse strain carrying a homozygous spontaneous missense mutation was reported to show skin manifestations similar to ichthyosis [Smyth et al., 2008]. Lipid analysis in $Abca12$ mutant epidermis revealed defects in lipid homeostasis, suggesting that $Abca12$ plays a crucial role in maintaining lipid balance in the skin [Smyth et al., 2008]. The cells from the $Abca12$ mutant mouse have severely impaired lipid efflux and intracellular accumulation of neutral lipids [Smyth et al., 2008]. Abca12 was also demonstrated as a mediator of Abca1-regulated cellular cholesterol efflux [Smyth et al., 2008]. Injection of a morpholino designed to target a splice site at the exon 4/intron 4 junction to block $Abca12$ pre-mRNA processing induced altered skin surface contours, disorganization of the melanophore distribution, pericardial edema and enlargement of the yolk sac at 3 days postfertilization in the larvae of the zebrafish. It was also associated with premature death at around 6 days postfertilization. These results suggest that $Abca12$ is an essential gene for normal zebrafish skin development and provide novel insight into the function of ABCA12 [reported at the Annual Meeting of the Society for Investigative Dermatology 2010; Abstract, Frank et al. J Invest Dermatol 2010;130:S86].

HI patients often die in the first 1 or 2 weeks of life. However, once they survive beyond the neonatal period, HI survivors' phenotypes improve within several weeks after birth. In order to clarify mechanisms of the phenotype recovery, we studied grafted skin and keratinocytes from $Abca12$-disrupted ($Abca12^{-/-}$) mouse [Yanagi et al., 2010]. $Abca12^{-/-}$ skin grafts kept in a dry environment exhibited dramatic improvements in all the abnormalities seen in the model mice. Increased transepidermal water loss, a parameter of barrier defect, was remarkably decreased in grafted $Abca12^{-/-}$ skin. 10 passage-subcultured $Abca12^{-/-}$ keratinocytes showed restoration of intact ceramide distribution, differentiation-specific protein expression, and profilaggrin/filaggrin conversion, which were defective in the primary culture [Yanagi et al., 2010]. These observations suggested that, during maturation, $Abca12^{-/-}$ epidermal keratinocytes regain normal differentiation processes, although the exact mechanisms of this restoration is still unknown [Yanagi et al., 2010].

We tried fetal therapy with systemic administration of retinoid or dexamethasone, which are effective treatments for neonatal HI and neonatal respiratory distress, respectively, to the pregnant mother mice; however, neither improved the skin phenotype or extended the survival period [Yanagi et al., 2008a]. Retinoids were also ineffective in in vivo studies using cultured keratinocytes from the model mice [Yanagi et al., 2010].

Prenatal Diagnosis of Harlequin Ichthyosis

In families with a history of HI, the parents' request for prenatal diagnosis is not easily ignored.

Before the causative gene for HI was identified, prenatal diagnosis had been performed by fetal skin biopsy and electron microscopic observation during the later stages of pregnancies at 19–23 weeks estimated gestational age for more than 20 years [Akiyama et al., 1994, 1999; Blanchet-Bardon et al., 1983; Shimizu et al., 2005]. The late timing of prenatal testing was a heavy burden on the pregnant mothers. In addition, when a fetus was diagnosed as affected, it was a major problem to induce a therapeutic termination at that late stage of pregnancy. After identification of $ABCA12$ as the causative gene for HI, it has become feasible to perform DNA-based prenatal diagnosis for HI by chorionic villus or amniotic fluid sampling at a much earlier

stage of pregnancy, with a significantly lower risk to fetal health and a reduced burden on mothers [Akiyama et al., 2007b]. Indeed, prenatal diagnosis and exclusion of HI by DNA testing were performed in our laboratory [Akiyama et al., 2007b; Yanagi et al., 2008b].

In the near future, it is hoped that much earlier prenatal diagnosis by completely noninvasive analysis of DNA from fetal cells in maternal circulation [Uitto et al., 2003] and preimplantation genetic diagnosis [Fassihi et al., 2006; Wells and Delhantry, 2001] will be available for HI.

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Invited review article

Extrinsic and intrinsic types of atopic dermatitis

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ABSTRACT

Atopic dermatitis (AD) can be categorized into the extrinsic and intrinsic types. Extrinsic or allergic AD shows high total serum IgE levels and the presence of specific IgE for environmental and food allergens, whereas intrinsic or non-allergic AD exhibits normal total IgE values and the absence of specific IgE. While extrinsic AD is the classical type with high prevalence, the incidence of intrinsic AD is approximately 20% with female predominance. The clinical features of intrinsic AD include relative late onset, milder severity, and Dennie-Morgan folds, but no ichthyosis vulgris or palmar hyperlinearity. The skin barrier is perturbed in the extrinsic, but not intrinsic type. Filaggrin gene mutations are not a feature of intrinsic AD. The intrinsic type is immunologically characterized by the lower expression of interleukin (IL) -4, IL-5, and IL-13, and the higher expression of interferon-γ. It is suggested that intrinsic AD patients are not sensitized with protein allergens, which induce Th2 responses, but with other antigens, and metals might be one of the candidates of such antigens.

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Contents

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1. History of extrinsic and intrinsic atopic dermatitis (AD)

AD is a clinically defined, chronic-intermittent, genetically predisposed, eczematous dermatitis that starts at infancy or early childhood. Although a large number of clinical, laboratory and experimental studies have been performed, the pathophysiology of AD remains to be elucidated, because AD has a variety of aspects in the causes and pathogenesis.

The clinical phenotype of AD has been classified into the extrinsic and intrinsic types [1]. Historically, this dichotomy was first used for asthma. The terminology of extrinsic or allergic asthma was first introduced by Rackeman in 1947 and referred to the triggering role of allergens in asthma. By symmetry, he described intrinsic or non-allergic asthma as a disease characterized by later onset in life, female predominance, higher degree of severity, and more frequent association with nasosinusal polyposis. As intrinsic asthmatic patients was not improved by conventional treatments, this author considered intrinsic asthma to be caused by a non-allergic, unknown phenomenon [2].

In AD, the extrinsic and intrinsic types began to be adopted in the late 1980s [3]. They are also called the allergic (or classical) and non-allergic types. Since there is still no sufficient consensus whether the intrinsic type is a distinct entity, some researchers denominate it atopiform dermatitis [4]. However, the classification into the extrinsic and intrinsic AD has been widely used especially since the millennium. Recently, various kinds of clinical studies have been performed under this dichotomy in many countries, including Germany [1,5,6], Netherland [4], Hungary [7], Italy [8,9] and other European countries, and Asian countries such as Korea [10,11], and Japan [12].

2. Definition

Extrinsic AD and intrinsic AD are defined according to IgE-mediated sensitization, namely the presence or absence of specific IgE for environmental allergens and food allergens [11,12,13]. According to the EAACI nomenclature task force, the term "atopic eczema/dermatitis syndrome (AEDS)" can be used to cover the different subtypes of AD. In this nomenclature, the intrinsic type is termed non-allergic AEDS, which shows normal IgE levels, no specific IgE, no association with respiratory diseases (bronchial asthma or allergic rhinitis), and negative skin-prick tests to common aeroallergens or food allergens [14]. Since total serum IgE values are significantly associated with the allergen-specific IgE status [15], total IgE can be regarded as a clinically useful parameter to differentiate between the extrinsic and intrinsic types in both adults [5,12] and children [15]. The reported mean values of total serum IgE in the intrinsic type are from 22.2 to 134 kU/l, or alternatively, IgE values less than 150 or 200 kU/l have been used for an indication of intrinsic AD [16]. Our study of Japanese patients also showed that the mean value of total serum IgE was 110.5 kU/l (11–219 kU/l) [12].

Among specific IgE antibodies, infantile AD patients are more allergic to food [11], while environmental antigens are common in adults. It should be careful that some allergens may not be useful to discriminate the two types. For example, IgE to *Malassezia sympodialis* was found in patients with the intrinsic type as well as the extrinsic type [17].

3. Prevalence of intrinsic AD

3.1. Incidence

Since extrinsic AD is the prototype of AD, its prevalence is well known. On the other hand, the frequency of intrinsic AD has been a matter of investigation. Schmid et al. [16] summarized the twelve reports that has been published from 1990 to 2000 and documented the clinical features of extrinsic and intrinsic AD. According to their review paper, the frequency of intrinsic AD was 10–45%. More recently, the incidence of extrinsic AD and intrinsic AD were reported as follows: 73% vs 27% [18] and 63% vs 37% [15] in German children, 88% vs 12% in Hungarian adults [7], 78.2% vs 21.8% in Dutch patients from 13 to 37 years of age [4], and approximately 80% vs 20% in Korean [19]. These data are in accordance with the empirical knowledge that about 20% of AD patients show normal IgE levels and lack of sensitization towards environmental allergens. Intrinsic AD is seen in various countries, but the prevalence may depend on local areas, as it was reported that intrinsic AD was higher in incidence in East Germany than West Germany, although the exact reason remains unclear [6].

3.2. Female predominance

The female predominance in intrinsic AD is well known and has been observed by a number of studies [1,4,16,20]. Our observation disclosed that 76.5% of AD patients were female [12]. More extremely, the 14 intrinsic AD patients enrolled in a study by another group were all female [20].

3.3. Adults and children

Several reports on the prevalence may provide an implication that the intrinsic type is seen at higher frequencies in children than adults [15]. A Korean group of AD investigators showed that the intrinsic type is more prevalent in infancy, and even the third group of the indeterminate type between the intrinsic and extrinsic ones can be identified in this younger generation [11]. A prospective birth cohort study followed for 5 years by a German group demonstrated that one third of child AD was the intrinsic one, and more common in female [21]. Another German group indicated the low prevalence of the intrinsic AD among adult patients [5]. They showed 6.9% patients fulfilled the criteria of intrinsic AD, and after follow-up, the incidence was declined to 5.4% because some patients developed respiratory allergies or IgE-mediated sensitizations. Taken together these observations, it is likely that the intrinsic type is more prevalent in children than adults.

4. Clinical features

The skin manifestations of the two types of AD are indistinguishable. As shown in Figs. 1–3, patients with the intrinsic type share the features with those with extrinsic type. However, Brenninkmeijer et al extensively studied the clinical features of intrinsic AD [4] and found that the Dennie-Morgan fold is significantly more present in the intrinsic type (Fig. 2). The later onset of disease and milder
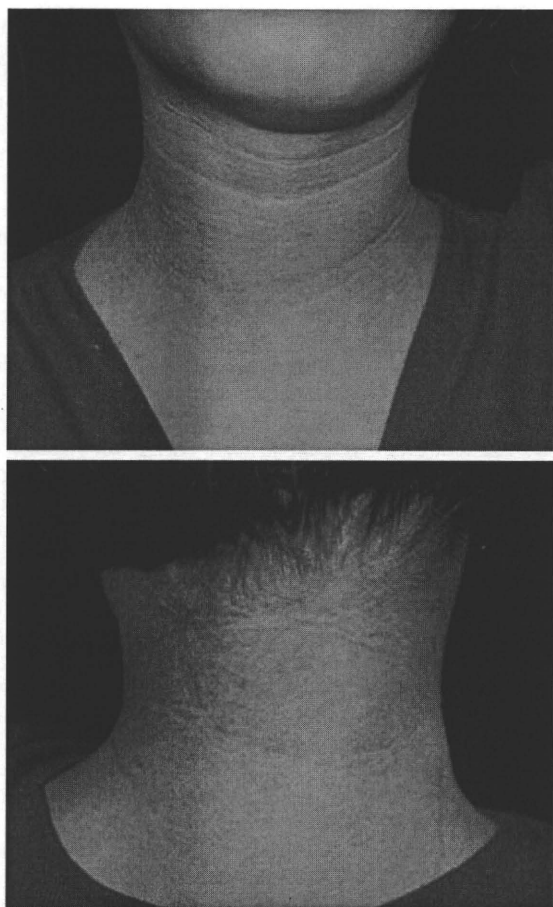
Fig. 1. Intrinsic AD. A 25-year-old female, with total serum IgE, 69 kU/l; and blood eosinophils, 10%. A lichenified eruption on the neck and upper chest (top) and nuchal area (bottom).

Fig. 3. Intrinsic AD. A 29-year-old female, with total serum IgE, 43 kU/l; and blood eosinophils, 11%. A lichenified eruption on the neck and upper chest (top) and upper back with scratches (bottom).

5. Skin barrier function

5.1. Barrier function of stratum corneum

The barrier function is usually assessed by transepidermal water loss (TEWL) and skin surface hydration (capacitance). The extrinsic AD patients showed increased TEWL and lower skin surface hydration, whereas the intrinsic patients showed no significant differences in TEWL or skin surface hydration as compared to control [19]. On the antecubital fossae, both types of AD patients showed higher TEWL and decreased capacitance. We examined the skin surface hydration and TEWL at the nonlesional forearm and lower leg of patients and normal volunteers in a comparison between the extrinsic and intrinsic types [12]. The level of skin surface hydration was significantly lower in extrinsic AD than in normal control subjects. On the other hand, there was

disease severity are also characteristics of intrinsic AD. The features that are negatively associated with intrinsic AD include personal or family history of atopy, recurrent conjunctivitis, palmar hyperlinearity, keratosis pilaris, pityriasis alba, non-specific hand or foot eczema, and influence of emotional or environmental factors [4]. As mentioned below, some of these non-associated features are considered to stem from the lack of barrier disruption and/or filaggrin gene mutations in intrinsic AD (Table 1).

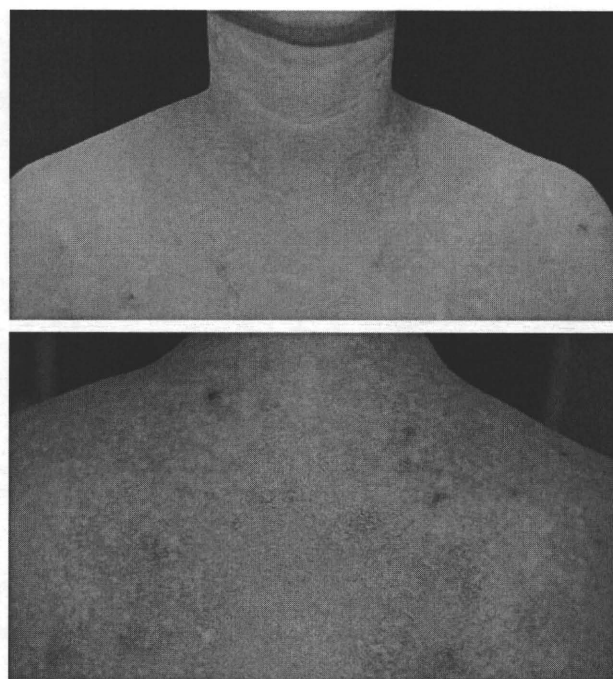Fig. 2. Intrinsic AD. A 32-year-old female, with total serum IgE, 226 kU/l; and blood eosinophils, 18%. A lichenified eruption with Dennie-Morgan folds on the lower eyelids and pigmentation on the lips (left); and a pigmented and chronic lesion on the antecubital fossa (right).

Table 1
Characteristics of intrinsic AD.

1. Definition Normal total serum IgE values (mean total serum IgE, 22.2–134 kU/l [1]) Absence of specific IgE for environmental allergens and food allergens
2. Incidence Percentage of intrinsic AD in total AD: 10–45% [1], 27% [2], 37% [3], 12% [4], 21.8% [5], 20% [6] Female predominance (collectively 70–80%) [1,5,7,8]
3. Clinical features Dennie-Morgan fold [5] No ichthyosis vulgris or palmar hyperlinearity [5] No non-specific hand or foot eczema [5] Lower colonization of *Staphylococcus aureus* [9] Relative late onset Milder severity
4. Skin barrier Normal barrier function [6,10] No filaggrin mutation (presence of filaggrin mutations in extrinsic AD [24,25])
5. Immunological features Lower expression of IL-4, IL-5, and IL-13 [11] Higher expression of IFN-γ [12]
6. Contact allergens High prevalence of metal allergy [39,40]

no significant difference in the hydration level between intrinsic AD and healthy control. The extrinsic type tended to be lower than the intrinsic type at both sites. Thus, the skin barrier function was impaired in extrinsic AD and preserved in intrinsic AD.

5.2. Pruritus perception and barrier function

The skin perception threshold of electric current stimuli is one of the indices of itch. We found that the electric current perception threshold was significantly correlated with the skin surface hydration and inversely with TEWL in intrinsic AD patients as well as healthy individuals. In contrast, extrinsic AD patients did not exhibit such a correlation. Therefore, intrinsic AD patients retain the normal barrier function and sensory reactivity to external pruritic stimuli [14].

5.3. Presence and lack of filaggrin mutations in extrinsic and intrinsic AD, respectively

The recent identification of loss-of-function mutations in filaggrin as a widely replicated major risk factor for eczema sheds new light on the mechanisms of AD [22].

These mutations also represent a strong genetic predisposing factor for atopic eczema, asthma and allergies in various countries [23]. Profilaggrin is the major component of the keratohyalin granules within epidermal granular cells. During epidermal terminal differentiation, the profilaggrin polyprotein is dephosphorylated and rapidly cleaved by serine proteases to form monomeric filaggrin, which is further degraded into natural moisturising factor. Recent human genetic studies strongly suggest that perturbation of skin barrier function as a result of reduction or complete loss of filaggrin expression leads to enhanced percutaneous transfer of allergens. Filaggrin is therefore in the frontline of defense, and protects the body from the entry of foreign environmental substances that can otherwise trigger aberrant immune responses. The association of the filaggrin mutations in particular with the extrinsic type of AD was observed [24,25]. Furthermore, filaggrin mutations are significantly associated with palmar hyperlinearity in patients with AD, which represents a shared feature of AD and ichthyosis vulgaris. This is in accordance

with the finding that palmar hyperlinearity is negatively associated in the intrinsic type [4]. In our preliminary study, we found that typical cases of intrinsic AD had no mutation in filaggrin, whereas some of the extrinsic patients possessed filaggrin mutations. Although future studies are necessary, it is expected that barrier disruption, as represented by filaggrin gene mutations, is associated with extrinsic AD.

6. Immunological characteristics of circulating T cells and cytokines/chemokines

6.1. Systemic Th1/Th2 immunological state

AD is well known as a Th2-polarized disease. However, there have been reported some differences in systemic cytokine polarization between the two types of AD. As expected with elevation of total serum IgE, extrinsic AD patients show high levels of Th2 cytokines, IL-4, IL-5 and IL-13, and intrinsic AD is linked with much lower levels of IL-4 and IL-13 [8]. Along with the elevation of IL-5 [26,27], eosinophil counts [11] and eosinophil cationic protein levels [18] are increased in the extrinsic type of AD. On the other hand, there was a report demonstrating that both extrinsic and intrinsic patients showed increased production of IL-5 and IL-13 [28]. In that study, however, when peripheral blood mononuclear cells were stimulated with anti-CD3 antibody, extrinsic AD patients had a decreased capacity to produce IFN-γ and GM-CSF as compared to the intrinsic AD [28]. Accordingly, we found, in our preliminary study, that there was no significant difference in the percentages of IL-4+ or IL-17+ T cells between the extrinsic and intrinsic types, but that of IFN-γ+ T cells was higher in the intrinsic type than the extrinsic type. Thus, there are some variations in these results of Th1 and Th2 cytokines, perhaps depending on the evaluation systems, i.e., measurements of cytokine protein amounts in either *in vivo* sera or *in vitro* culture supernatants, mRNA expression by lymphocytes, and intracellular cytokine staining in T cells. However, all the data can be interpreted to indicate that the extrinsic pathogenetic factors mount a Th2-skewing action, and that the intrinsic type shows less Th2-skewing state or relative overproduction of Th1 cytokine IFN-γ.

6.2. Chemokines and others

With regard to chemokines, patients of both types showed high serum amounts of CCL17/TARC and CCL22/MDC and high peripheral blood mononuclear cell expression of CCL17 and CCL22 at comparable levels [29]. Therefore, no difference was observed in the promoted production of chemokines attractive to Th2 cells. The blood levels of soluble receptors derived from lymphocytes correlate to the activity in various diseases. There is no significant difference in the elevated amounts of sCD23, sCD25, and sCD30 between the two types [30].

7. Immunological characteristics of skin lesions

7.1. T cells and cytokines

In skin lesions, CD4+ T cells, CD8+ T cells, and Langerhans cells are comparably increased in both extrinsic and intrinsic AD, but eosinophils infiltrate in the dermis more markedly in the extrinsic than the intrinsic type, and the extrinsic type exhibits more prominent deposition of eosinophil granular protein and higher staining for eotaxin [10,31]. Although the levels of mRNA expression for IL-5, IL-13, and IL-1β are higher in both types of AD patients than non-atopic subjects, extrinsic AD shows even higher levels than intrinsic AD [31]. The expression of IFN-γ, IL-12, and GM-CSF, IL-4, and IL-10 are elevated in both types without

differences between the extrinsic and intrinsic AD [31]. Thus, tissue eosinophilia and IL-5 expression may be a characteristic of the extrinsic type.

7.2. Dendritic cells (DC) and Langerhans cells (LC)

As to epidermal DC, the extrinsic type is characterized by a significantly high level of the expression of IgE high-affinity receptor (FCεR) on the CD1a$^+$ epidermal DC compared to the intrinsic type [1,32]. When the high-affinity/low-affinity expression ratio is used as a disease marker for AD, the values for intrinsic AD fall below the diagnostic cut-off level, suggesting that intrinsic AD can be distinguished by phenotyping of epidermal DC [1,32]. In accordance with these data from the lesional skin, the surface expression of the high- and low-affinity receptor for IgE and the IL-4Rα chain are significantly elevated in monocytes from patients with the extrinsic type [2]. As described below, it is possible that epidermal LC in the barrier-disrupted skin produce high amounts of Th2 and eosinophil chemokines, further suggesting that LC are activated in the extrinsic type.

8. Relationship between barrier status and skin immune reactions

8.1. Epidermal cytokine production in barrier-disrupted skin

The skin immune status is closely associated with the disordered condition of skin barrier (Fig. 4). Studies using a mouse model of contact hypersensitivity (CHS) have shown that CHS responses to hapten are increased when a hapten is applied to the barrier-damaged skin [33]. Barrier disruption of the skin is experimentally performed by extraction of epidermal lipids with acetone or removal of corneocytes by tape stripping. Both procedures can induce elevated CHS responses. Not only increased permeability of hapten through the epidermis but also altered immune functions of epidermal cells potentiate T-cell activation in acute barrier disruption [33]. Such augmentation of immune reactivity may be critical to elimination of environmental noxious agents that penetrate easily into the barrier-disrupted epidermis, and it is also closely related to the mechanism underlying extrinsic AD.

8.2. Epidermal chemokine production in barrier-disrupted skin

Regarding epidermal chemokines of the barrier-disrupted skin, the mRNA expression levels of Th1 chemokines (CXCL10, CXCL9 and CXCL11), Th2 chemokines (CCL17 and CCL22) and eosinophil chemoattractant (CCL5) are high in the epidermal cells from BALB/c mice. In particular, we found that CCL17, CCL22 and CCL5 were remarkably elevated in BALB/c mice [34]. Tape stripping induced dermal infiltration of eosinophils in BALB/c mice, and the late-phase reaction was increased with infiltration of Th2 cells as well as eosinophils, when challenged via the tape-stripped skin. Notably, Th1 chemokines (CXCL9 and CXCL10) and Th2 chemokines (CCL17 and CCL22) are derived mainly from keratinocytes and LC, respectively [35]. In this notion, one of the crucial actions of IFN-γ is upregulation of keratinocyte production of Th1 chemokines and downregulation of LC production of Th2 chemokines. Therefore, the barrier damage likely induces the infiltrates of Th2 cells and eosinophils in extrinsic AD, but their infiltrates are inhibited by IFN-γ in intrinsic AD.

8.3. Implications for the difference between extrinsic and intrinsic AD

The above findings suggest that Th2 and eosinophil responses and resultant late-phase reaction are prone to take place in the skin with damaged barrier by the modulated function of LC. This may provide the mechanism of Th2-polarized immunophenotype of the extrinsic AD. On the contrary, LC are not stimulated to produce Th2 chemokines in the intrinsic type because of the presence of normal stratum corneum. Protein antigens penetrating the damaged barrier further induce the Th2-shifted response in the extrinsic AD, while non-protein antigens exert the Th1 response as well in the intrinsic AD (Fig. 4).
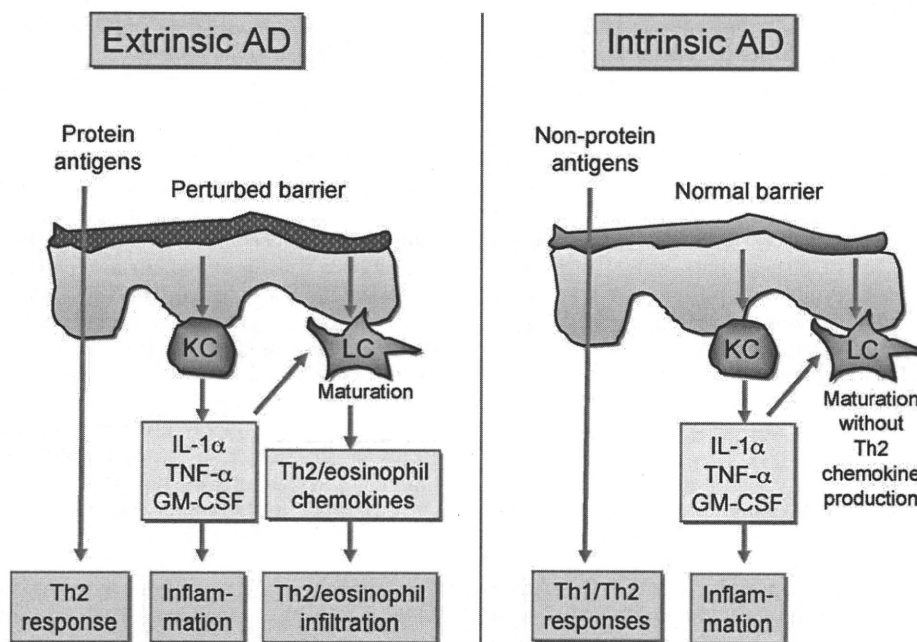
Fig. 4. Comparison between extrinsic and intrinsic AD in relation to the barrier and immune states.
Protein and non-protein antigens are causative in the extrinsic and intrinsic types, respectively. In both types, antigen application to the skin stimulates keratinocytes to produce cytokines, including IL-1α, TNF-α, and GM-CSF, which induce maturation of Langerhans cells (LC). In the perturbed skin of extrinsic AD, LC can produce CCL17/TARC, CCL22/MDC, and CCL5/RANTES, which promote infiltration of Th2 cells and eosinophils. On the other hand, LC of the intrinsic AD do not elaborate those chemokines.

It has been reported that Th2 cytokine IL-4 suppresses the enhancement of ceramide synthesis and cutaneous permeability barrier functions, which further aggravates the barrier [36]. This 'outside-to-inside, back to outside' paradigm [37] is applicable for the pathogenesis of extrinsic AD. A more recent observation suggests that neutralization of the normally acidic stratum corneum has deleterious consequences for permeability barrier homeostasis and stratum corneum integrity/cohesion attributable to serine proteases activation leading to deactivation/degradation of lipid-processing enzymes and corneodesmosomes [38]. Hyperacidification improves permeability barrier homeostasis, attributable to increased activities of two key membrane-localized, ceramide-generating hydrolytic enzymes, which correlate with accelerated extracellular maturation of stratum corneum lamellar membranes. Thus, the surface pH may be another important factor to differentiate between the extrinsic and intrinsic types.

9. Patch tests and metal allergy

9.1. Patch tests for mite antigens

An Italian group performed patch test with house dust mites at a concentration of 20% in petrolatum in the extrinsic and intrinsic types of adult male AD patients [9]. The patch test was positive in 47.4% of extrinsic AD and in 66.6% of intrinsic AD, and in 12.2% of healthy subjects [9]. Since that extrinsic AD patients usually have high levels of IgE specific for mites, the authors wondered the reason why the patch test was highly positive in the intrinsic AD. However, patch tests can reflect mostly the T-cell mediated contact sensitivity, and the IgE-high extrinsic nature does not promote the patch test reactions. Rather, given that IFN-γ is produced at a higher level in the intrinsic type than the extrinsic type, the higher frequency of positive reaction in the intrinsic type seems to be reasonable.

9.2. Patch tests for metals

It is known in patients with AD that the most frequent contact allergens are metals [39]. In 137 atopic children, 19.3% patients were positive to metals [39]. In 1965, Shanon reported that patients with metal allergy occasionally exhibit a skin manifestation indistinguishable from AD under the name of "pseudo-atopic dermatitis" [40,41], and chrome is the causative in their report [40]. Some patients with AD were improved by intake of metal-free diet and elimination of metals [41]. We found that patients with intrinsic AD showed positive patch tests to cobalt, chrome, and/or nickel at a higher percentage than extrinsic one, suggesting that systemic metal allergy is one of the potential causes of intrinsic AD. With regard to metals, our tentative observation with sweat demonstrated that a high incidence of sweat allergy in AD and a therapeutic effect of desensitization with sweat in the patients. Since sweat contains high concentrations of metals, this finding might be related to the pathogenetic role of metals in intrinsic AD.

10. Skin infections

Both extrinsic and intrinsic AD patients suffer from recurrent bacteria and viral infections [42]. A higher colonization of *Staphylococcus aureus* was observed in the extrinsic (71%) *vs* the intrinsic children (49%) [43]. The expression of human β-defensin-3, an anti-microbial peptide, is decreased in both types of AD as compared to normal skin and psoriatic skin [42]. Therefore, skin infection with microorganisms, in particular *S. aureus*, may be severer in the extrinsic type because of barrier perturbation, but it remains unclear whether or not the defense responses are different between the types.

11. Neurotrophins and neuropeptides

Neurotrophins, nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), are increased in both extrinsic and intrinsic AD, suggesting a similar pathophysiologic background implicating a neuroimmune network [27]. However, there is a significant correlation between BDNF and SCORAD only in the intrinsic type [27]. Maternal NGF levels were significantly higher in patients with both extrinsic and intrinsic AD than controls [30]. It is an issue to be elucidated whether these neurotrophines or neuropeptides such as substance P are different between the two types.

12. Animal models for intrinsic AD

A non-IgE-associated AD model was regarded as a mode of human intrinsic AD [44]. In an animal model of AD, IL-18 contributes the spontaneous development of AD-like skin lesions independently of IgE [45]. When the skin barrier was destroyed in mice and protein A from *S. aureus* was topically applied to the skin, the mice developed AD lesions with dermal infiltration of eosinophils and mast cells and showed an increase in serum levels of IL-18, but not IgE [46]. In this model, IL-18 might be important for the development of infection-associated AD by induction of IL-3 from IFN-γ- and IL-13-producing "super" Th1 cells. Since the intrinsic AD shows high levels of IFN-γ-producing cells [28] and normal levels of IgE, this mouse model resembles intrinsic AD and suggests that some intrinsic AD patients may be related to infection.

13. Conclusions

The causes and mechanisms of intrinsic AD remain unfully elucidated. However, as compared to extrinsic AD, the intrinsic type can be characterized by normal barrier function [12] and IFN-γ-producing potency [28]. These findings suggest that the intrinsic AD patients are not sensitized with protein allergens, which induce Th2 responses, but with other antigens. Metals might be one of the candidates as antigens [40].

Some dermatologists are still skeptical whether the distinction between the intrinsic and extrinsic types of AD is really meaningful. If the contactants and pathophysiology of intrinsic AD are clarified, we might eliminate the term of "intrinsic" from the whole spectrum of AD. However, we have been unable to elucidate them to date. Furthermore, as shown in Figs. 1–3, the clinical appearance of intrinsic AD resembles that of extrinsic AD, expect for a small group of intrinsic patients. In this context, it is reasonable to use "intrinsic" in clinical dermatology.

The extrinsic nature may be changed as the patients grow. Therefore, the classification into the extrinsic and intrinsic types is necessary at each stage of life, i.e., infancy, childhood, teenage, and adult for the allergological management of patients as to allergen avoidance, second allergy prevention, and immunotherapy [14]. However, the risk of an "atopy march" is significantly lower in children with the intrinsic type [14].

A German study demonstrated that the intrinsic type was associated with early daycare attendance [21]. In relation to the feasibility of AD in individuals, early daycare attendance is known as a factor related to the hygiene hypothesis as well as the number of older siblings of individuals. Therefore, intrinsic AD is different from extrinsic AD, whose development is depressed by Th1-inducing environmental factors. Again, it appears that the intrinsic type is not related to the pure Th2 dominant immunological state. Future studies on the intrinsic type of AD may clarify the pathophysiology of not only intrinsic AD, but also dermatitis of

unknown cause that have been called atopiform dermatitis [4] or pseudo-atopic dermatitis [40].

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This is the first report of excessive skin tissue destruction after topical contact with *Dieffenbachia* guttation fluid although *Dieffenbachia* intoxication occurs frequently with oral intake.[6] Hitherto, only local irritation with dermatitis has been reported after skin contact.[2,6] Except for fingertip rhagades, our patient was healthy and a nonsmoker. The intact cornified layer is an effective barrier against plant poisons, but small lesions permit penetration followed by severe necrosis. Biochemical analyses confirmed that the newly identified MMP-3-like metalloproteinase could have caused the tissue damage in the present case. Many plants, including *Dieffenbachia*, excrete superfluous liquid from the leaves, which contains proteins.[7] Obviously, *Dieffenbachia* produces a protective enzyme cocktail that is secreted into the guttation fluid.

MMPs belong to the superfamily of metzincins, and there are only a few characterized plant metalloproteinases.[8] In previous studies, a trypsin-like proteinase named 'dumbcain',[9] not oxalate crystals, was accused of tissue damage.[3] The proteinase isolated here showed different collagen fragments after degradation compared with trypsin and belongs to the metalloproteinase family, as assessed by ETD-MS. Snake venom contains metalloproteinases with structural and functional homology to mammalian MMP and similar severe tissue necrosis after a snake bite.[10] Biochemical analyses and the presented clinical case support the assumption that the isolated *Dieffenbachia* MMP-3-like proteinase caused tissue destruction and necrosis.

In conclusion, *Dieffenbachia* is a beautiful plant with wide distribution as a room decoration. Its sap contains a poisonous metalloproteinase that can cause tissue necrosis after local skin contact. Immediate radical debridement in combination with flap surgery stopped disease progress. To avoid tissue damage use of protective gloves while handling this plant is recommended.

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Expression of Snail1 in the fibrotic dermis of postmenopausal frontal fibrosing alopecia: possible involvement of an epithelial–mesenchymal transition and a review of the Japanese patients

MADAM, Postmenopausal frontal fibrosing alopecia is characterized by a progressive frontotemporal recession due to a scarring hair loss.[1] Histological examination shows a reduction or loss of terminal hair follicles and a replacement by fibrosis around the affected hair follicles. Lymphocytic infiltration is usually observed in a lichenoid pattern in the upper dermis. Although postmenopausal frontal fibrosing alopecia is considered as a variant of lichen planopilaris, its exact pathogenesis still remains to be clarified. We report a patient with postmenopausal frontal fibrosing alopecia showing expression of Snail1 in the fibrotic dermis, suggesting a role for epithelial–mesenchymal transition in the pathogenesis.

A 77-year-old Japanese woman was referred to us with a 4-month history of frontotemporal recession. She had applied betamethasone butyrate propionate lotion for a few months without any apparent hair regrowth. Clinical examination showed frontotemporal recession with uniformly pale skin (Fig. 1a,b). There was no change in her occipital hairs, eye-
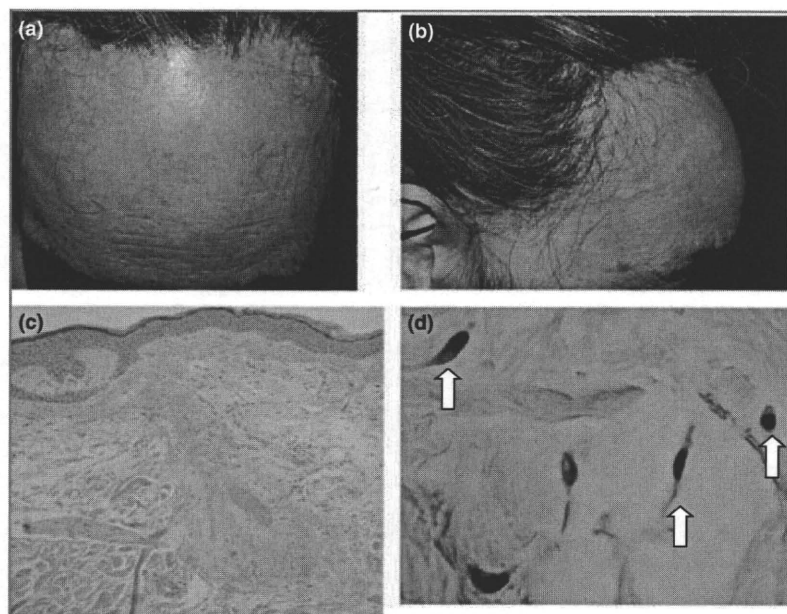
Fig 1. (a,b) Frontotemporal recession with uniformly pale skin. (c) Histology of a biopsy specimen taken from the temporal region. An arrector pili muscle inserting into the fibrotic tissue is surrounded by sparse lymphocytes (haematoxylin and eosin). (d) Snail1-positive cells found in the fibrotic dermis (arrows).

Table 1 Summary of 11 Japanese patients with postmenopausal frontal fibrosing alopecia

Patient	Age (years)	Duration (years)	Width (cm)	Progression	Eyebrow involvement	Response to steroid ointment	Reference
1	65	2	4.5–6	+	+	−	2
2	70	4	ND	ND	ND	ND	3
3	61	ND	3	ND	ND	ND	4
4	65	ND	3	ND	ND	ND	4
5	54	ND	3	ND	ND	ND	4
6	62	3	ND	−	−	−	5
7	72	ND	4	−	ND	ND	6
8	63	3	ND	ND	+	ND	7
9	66	6	ND	+	−	−	8
10	71	2	ND	−	−	−	9
11	77	0.3	2	−	−	−	This case

ND, not described.

brows, eyelashes, nails or buccal mucosa. Histopathology of a biopsy specimen taken from the temporal region showed an arrector pili muscle inserting into the fibrotic tissue surrounded by sparse lymphocytes (Fig. 1c). Blood examination revealed no abnormal findings. Based on the clinical and histological findings, we diagnosed her as having postmenopausal frontal fibrosing alopecia. Without any treatment the alopecia stopped progression, but currently it has shown no improvement.

In Table 1 we summarize the clinical data of 11 Japanese patients with postmenopausal frontal fibrosing alopecia.[2-9] Except for patients 9 and 11, all reports were written in Japanese. In comparison with English patients with postmenopausal frontal fibrosing alopecia,[10] this literature review revealed that the age at onset is not significantly different between Japanese (59–77 years, mean 63.1) and English patients (34–71 years, mean 60.1). Eyebrow involvement was reported in only two of six Japanese patients (33%), while lost or sparse eyebrows were found in 12 of 14 English patients (86%). No lichen planus lesions at sites other than the head and eyebrows were reported in Japanese patients, whereas the coexistence of lichen planus at the oral or vulval region was seen in three of 15 English patients (20%). Thus, postmenopausal frontal fibrosing alopecia is more severe, with more accompanying lesions, in caucasian than in Japanese subjects.

To explore the pathomechanism of fibrosis in this disease, we performed immunohistochemistry using an anti-Snail1 antibody (Abgent, San Diego, CA, U.S.A.), a useful marker for epithelial–mesenchymal transitions.[11] Snail1-positive cells were

found only in the fibrotic dermis of the patient (Fig. 1d). It had been thought that fibrosis occurs by the pathological activation of interstitial fibroblasts and the resultant conversion to myofibroblasts producing the fibrotic collagen network. In renal fibrosis, however, elegant cell tracing studies have shown that a significant portion of these myofibroblasts arises from the conversion of epithelial cells through an epithelial–mesenchymal transition process.[12] Moreover, epithelial–mesenchymal transition also occurs during liver fibrosis and pulmonary fibrosis. The existence of Snail1-positive cells in the fibrotic dermis of our patient suggests that the fibroblasts are in part derived from the hair follicle cells via an epithelial–mesenchymal transition process. As transforming growth factor-β is a well-known inducer of this transition, it might be a target for a new therapeutic approach to postmenopausal frontal fibrosing alopecia in the future.

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Frontal fibrosing alopecia in two sisters

MADAM, Frontal fibrosing alopecia (FFA) is a cicatricial alopecia first described by Kossard in 1994[1] and characterized by a progressive and symmetrical band of frontal or frontoparietal hair recession with loss of follicular orifices and perifollicular erythema around the remaining hairs. The eyebrows are frequently involved, with partial or complete absence of hair.[2] The condition has been reported mainly in postmenopausal caucasian women and all reported cases were sporadic.[3] We report two sisters with postmenopausal FFA. This is the first familial report of the condition.

Patient 1, a 65-year-old woman, was first diagnosed with FFA in our department at the age of 57 years (Fig. 1a). She was treated with oral finasteride 2·5 mg daily and topical steroids, with slow progression of the disease (Fig. 1b). In October 2009 she noticed that her sister was developing a similar pattern of hair loss and brought her for consultation in our department.

Patient 2, a 59-year-old woman, the sister of patient 1, had a 1-year history of progressive alopecia involving her frontotemporal scalp (Fig. 2a). Dermoscopy showed perifollicular erythema and scaling (Fig. 2b). Clinical history revealed that the patient had undergone oophorectomy at the age of 32 years and had a surgical resection of a colorectal adenocarcinoma followed by chemotherapy at the age of 54 years. At the age of 55 years she was treated with interferon and ribavirin for 8 months because of hepatitis C.
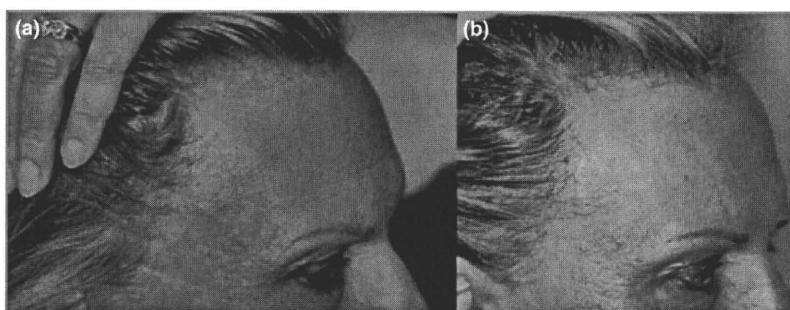

Fig 1. Patient 1. (a) Frontoparietal hairline recession at the first visit. (b) Lack of hair with a pale and shining skin on the frontoparietal hairline, resembling cicatricial alopecia.

Original Article

Tumor Cell Expression of Programmed Cell Death-1 Ligand 1 Is a Prognostic Factor for Malignant Melanoma

Ryosuke Hino, MD[1]; Kenji Kabashima, MD[1,2,3]; Yu Kato, MD[4]; Hiroaki Yagi, MD[5]; Motonobu Nakamura, MD[1]; Tasuku Honjo, MD[4]; Taku Okazaki, MD[4,6]; and Yoshiki Tokura, MD[1]

BACKGROUND: Melanoma tends to be refractory to various immunotherapies because of tumor-induced immunosuppression. To investigate the mechanism underlining the immunosuppression of melanoma patients, the authors focused on programmed cell death-1 (PD-1)/PD-1 ligand 1 (PD-L1) interaction between tumor cells and T cells. **METHODS:** Melanoma specimens were collected from 59 primary tumors, 16 lymph nodes, and 4 lesions of in-transit metastasis. Specimens stained with anti-PD-L1 monoclonal antibodies were digitalized to jpg files. To evaluate the intensity of PD-L1 expression, histograms were used, and the red density (RD) was measured. PD-1 expression on T cells was analyzed in blood samples from 10 patients who had stage IV melanoma and in 4 samples of in-transit metastases. **RESULTS:** Twenty-five patients comprised the "low" PD-L1 expression group (RD value, <90), and 34 patients comprised the "high" group (RD value, ≥90). Breslow tumor thickness in the high-expression group was significantly higher than in the low-expression group. Univariate and multivariate analyses revealed that the overall survival rate of the high-expression group was significantly lower than that of the low-expression group. In all patients with stage IV disease who were examined, both CD8-positive and CD4-positive T cells had significantly higher PD-1 expression levels in the peripheral blood. Tumor-infiltrating T cells expressed high levels of PD-1, and its expression was elevated further during the clinical course. **CONCLUSIONS:** The current results indicated that there is a correlation between the degree of PD-L1 expression and the vertical growth of primary tumors in melanoma. Multivariate analysis demonstrated that PD-L1 expression is an independent prognostic factor for melanoma. *Cancer* 2010;116:1757-66. © *2010 American Cancer Society.*

KEYWORDS: melanoma, peripheral blood mononuclear cells, programmed cell death, tumor-infiltrating lymphocytes.

Although malignant melanoma is a representative immunogenic tumor among various neoplasms,[1] it tends to be refractory to immunotherapy.[2,3] The presence or absence of tumor-infiltrating lymphocytes is 1 of several hallmarks that predict prognosis for patients with melanoma.[4] High frequencies of tumor-infiltrating, CD8-positive lymphocytes that are specific to melanoma antigens can be identified at tumor sites[5] or in peripheral blood from patients.[6] Conversely, an immunosuppressive status often is observed in patients with advanced malignant melanoma,[7] and many immunotherapies have been unsuccessful because of such immunosuppression. The number or function of CD4-positive/CD25-positive/ forkhead box P3 (Foxp3)-positive regulatory T (Treg) cells[8-11] or interleukin 10 (IL-10)-producing immunosuppressive dendritic cells[12-14] is increased or promoted during the progression of malignant melanoma, even when patients receive some tumor vaccination therapies.[3] Investigation of the mechanisms underlying this tumor-induced immunosuppression may provide clues about how to overcome malignant melanoma therapeutically.

Recently, it has been established that programmed cell death-1 (PD-1), an immunoinhibitory receptor that belongs to the CD28 family, plays a critical role in tumor immune escape.[15,16] Two ligands for PD-1, PD-1 ligand 1 (PD-L1) and

PD-1 ligand 2 (PD-L2) (also known as B7-DC) are involved in the negative regulation of cellular and humoral immune responses by engaging the PD-1 receptor.[15] PD-L1 is expressed on resting T cells, B cells, dendritic cells, macrophages, and parenchymal cells, including vascular endothelial cells and pancreatic islet cells.[15,16] Conversely, the expression of PD-L2 is limited to macrophages and dendritic cells.[17] Previous studies have demonstrated that PD-1/PD-L interaction inhibits T-cell growth and cytokine secretion[18] and that tumor cell-borne PD-L1 induces the apoptosis of tumor-specific T-cell clones in vitro,[19] suggesting the potential involvement of PD-Ls in tumor immunity. The expression of PD-L1 in tumors has been reported in melanoma[19,20]; in cancers of the lung,[19] breast,[21] ovary,[22] kidney,[23] pancreas,[24] esophageus,[25] and bladder[26]; and even in adult T-cell leukemia/lymphoma.[27] In addition, the involvement of PD-L1 has been demonstrated in the protection of cancer cells from cell lysis by activated T lymphocytes.[28] However, the expression of PD-L1 on melanoma cells in relation to tumor cell behavior and prognosis remains to be elucidated. In the current study, we investigated PD-L1 expression in human malignant melanoma to define its clinical significance and relevance to the prognosis for patients with these tumors.

MATERIALS AND METHODS

Patients and Samples

Patients who were enrolled in this study were treated and followed from 2000 to 2007 by the Department of Dermatology, University of Occupational and Environmental Health (Kitakyushu, Japan). Tumors were classified according to the American Joint Committee on Cancer (AJCC) staging system.[29] Patients were followed at regular intervals for evaluation of recurrence by physical examination and radiologic studies. Melanoma specimens were collected from 59 primary tumors, 16 lymph nodes (LNs) (9 metastatic LNs and 7 nonmetastatic LNs), and 4 in-transit metastases.

Each specimen was fixed with 20% formalin and embedded in paraffin, and serial sections were stained with hematoxylin and eosin for histologic evaluation. The specimens were digitized by using the NanoZoomer Digital Pathology C9600 (Hamamatsu Photonics, Hamamatsu, Japan), and Breslow tumor thickness (BTT) was analyzed with NDP View software (Hamamatsu Photonics).

Table 1. Relation Between Programmed Cell Death-1 Ligand 1 Expression in Primary Tumors and Other Clinicopathologic Factors

Variable	No. of Patients		P
	Low Expression	High Expression	
Total no.	25	34	
Sex			
Men	15	23	.5981[a]
Women	10	11	
Age: Mean±SD, y	68.84±2.85	69.94±2.1	.7509[b]
Tumor type			
NM	4	6	
ALM	18	21	
SSM	3	5	
LMM	0	2	
Tumor site			
Extremity	20	26	
Trunk	5	4	
Head and neck	0	4	
BTT: Mean±SD	1.92±0.42	3.1±0.33	.0298[b]
Tumor classification			
T0-T2	16	9	
T3-T4	9	25	.0072[a]
Ulceration			
Absent	19	22	
Present	6	12	.4031[a]
LN metastasis			
N0	22	21	
N1-N3	3	13	.0375[a]
Clinical stage			
0	5	3	
IA	5	1	
IB	5	4	
IIA	2	6	
IIB	3	4	
IIC	2	3	
IIIA	0	3	
IIIB	2	3	
IIIC	0	7	
IV	1	0	

SD indicates standard deviation; NM, nodular melanoma; ALM, acral lentiginous melanoma; SSM, superficial spreading melanoma; LMM, lentigo maligna melanoma; BTT, Breslow tumor thickness; LN, lymph node.
[a]Fisher exact test.
[b]Student t test for unpaired data.

Immunohistochemistry

Immunohistochemical staining for PD-L1 was achieved by using a monoclonal antibody (MoAb) capable of detecting PD-L1 on formalin-fixed, paraffin-embedded specimens.[22] Sample specimens were cut into 4-mm-thick sections that were deparaffinized in xylene (3 times for 10 minutes each) and dehydrated through graded alcohols
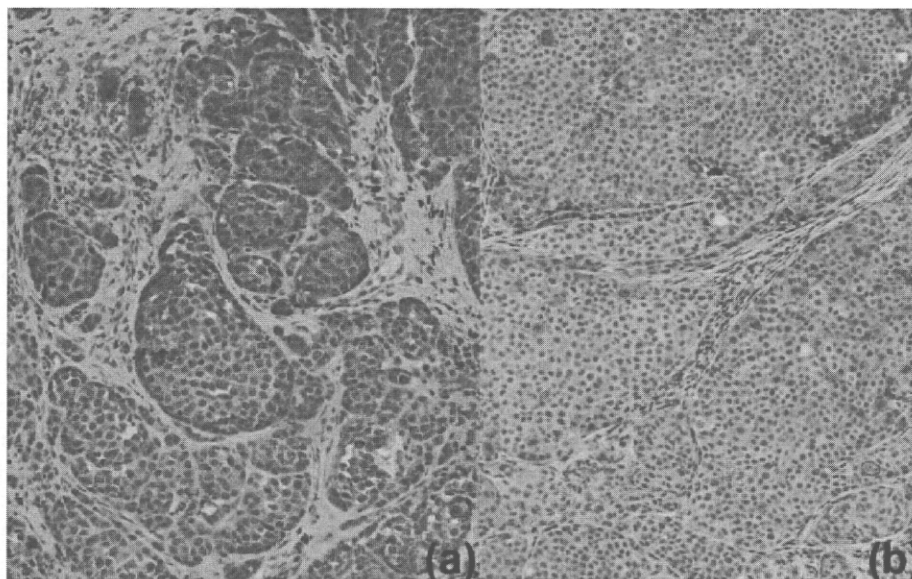
Figure 1. These photomicrographs illustrate immunohistochemical staining of programmed cell death-1 ligand 1 (PD-L1) in malignant melanoma. (a) An acral lentiginous melanoma on the left first toe in a human aged 78 years had a Breslow tumor thickness (BTT) of 5 mm and a red density (RD) of 26.57. (b) A nodular melanoma on the back of a human aged 72 years had a BTT of 2.8 mm and an RD of 153.81.

(99%, 99%, and 70%) to water. Antigens were retrieved by boiling in citrate buffer, pH,6.0, using microwaves. To block endogenous peroxidase activity, all sections were treated with 100% methanol containing 0.3% H_2O_2 for 15 minutes. Nonspecific binding of immunoglobulin G was blocked by using normal rabbit serum (Nichirei, Tokyo, Japan). The sections were incubated with mouse anti-PD-L1 MoAbs (clone 27A2; MBL, Nagoya, Japan)[22] overnight at 4°C. Then, they were incubated with biotinylated rabbit-antimouse secondary antibody (Nichirei) and subsequently incubated in a streptavidin-peroxidase complex solution for 30 minutes. Signals were generated by incubation with 3-amino-9-ethyl carbazole. Finally, the sections were counterstained with hematoxylin.

Analysis of Expression Intensity in Histologic Specimens

Digitized specimens were exported to JPG files by using NDP View software (Hamamatsu Photonics). The following processes were performed in Adobe Photoshop CS (J) (Adobe Systems, Inc. San Jose, Calif). Three different areas from the tumor cell cytoplasm were selected and expressed as Red channel histograms. In the bar graphs that were produced, the horizontal and vertical axes represented tone and quantity, respectively. Histograms revealed 255 different shades from pitch black (0) to pure white (255), and a number represented the level of brightness of each color. We analyzed the mean intensity of the histogram in the cytoplasm and averaged the value of 3 different areas. To obtain density, we calculated the 255-"mean" of each color. We called these values "red density"(RD) values and used them for further investigation. Specimens with an RD value <90 were defined as the low expression group, and specimens with an RD value ≥90 were defined as the high expression group.

Flow Cytometric Analysis

Blood samples were collected from 10 patients who had stage IV melanoma and from 5 normal, healthy volunteers to evaluate PD-1 expression on T cells. Peripheral blood mononuclear cells (PBMCs) were isolated from the heparinized venous blood of patients by using Ficoll-Hypaque (Sigma Chemical Company, St. Louis, Mo) density-gradient centrifugation. Four local metastatic skin lesions were removed surgically, dissociated by teasing, and subjected to flow cytometric analysis. Single cell suspensions were obtained from the excised metastatic skin tumors by teasing and filtering and were subjected to flow cytometric analysis. Cells were double stained with fluorescein isothiocyanate-conjugated anti-PD-1, anti-PD-L1, or anti-PD-L2 MoAb and phycoerythrin (PE)-conjugated anti-CD4 or anti-CD8 MoAb (all from BD Biosciences, San Diego, Calif) at 2 μg/10^6 cells in Hanks balanced salt solution containing 0.1%
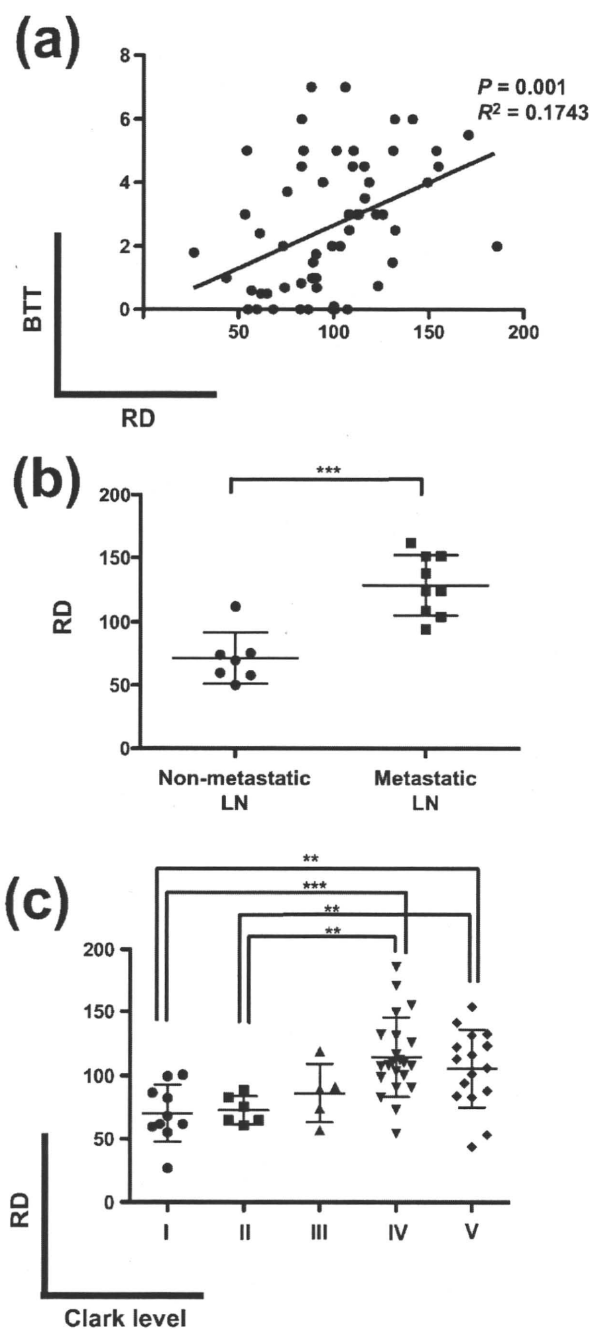
Figure 2. Correlations of programmed cell death-1 (PD-1) expression levels with Breslow tumor thickness (BTT), lymph node (LN) metastasis, and Clark level are illustrated. (a) A linear correlation was observed between red density (RD) and BTT. (b) In this comparison, RD in resected LNs was analyzed using an analysis of variance (ANOVA) between 2 groups: 1 with and 1 without LN metastasis. Error bar represents the mean ± standard deviation. (c) In this comparison, RD was analyzed by ANOVA among 5 groups with different Clark levels. Error bars represents the mean ± standard deviation; double asterisks, $P < .01$ (between the means); triple asterisks, $P < .001$ (between the means).

NaN_3 and 1% fetal calf serum. After incubation for 30 minutes at 4°C with MoAbs or isotype-matched controls, cells were washed twice and analyzed on a FACSCanto (Becton Dickinson, Mountain View, Calif). The mean fluorescence intensity (MFI) was calculated on a log scale.

Statistical Analyses

Fisher exact tests, chi-square tests, and Student t tests for unpaired data were used to analyze the association between PD-L expression and various clinicopathologic factors. The Pearson coefficient test was used to evaluate the correlation between RD and BTT. Univariate analyses of overall survival and progression-free survival were conducted with the log-rank test, and Kaplan-Meier curves were generated. Overall and progression-free survival was calculated from the date of operation to the date of first recurrence, death, or last follow-up. Multivariate comparisons were made using the Cox proportional hazards model. Except for the Cox multivariate analysis, every analysis was performed by using GraphPad Prism 5.0 software (GraphPad Software, Inc., San Diego, Calif). The Cox multivariate analysis was performed by using the JMP 5.0.1J software package (SAS Institute, Cary, NC). All P values $<.05$ were considered statistically significant.

RESULTS

Clinical Patient Profiles

The clinical characteristics of 59 patients (ratio of men to women, 38:21) are summarized in Table 1 in relation to expression levels of PD-L1 in tumor cells (low and high). The average patient age was 69.47 years (range, 25-87 years; standard deviation, 13 years). The most common site of melanoma was the extremity (78%), followed by the trunk (15.3%), and head and neck (6.8%). Thirty-nine patients were diagnosed with acral lentiginous melanoma, 10 patients were diagnosed with nodular melanoma, 8 patients were diagnosed with superficial spreading melanoma, and 2 patients were diagnosed with lentigo maligna melanoma. According to the AJCC staging system,[29] 8 patients (13.6%) had stage 0 melanoma, 6 patients (10.2%) had stage IA melanoma, 9 patients (15.3%) had stage IB melanoma, 8 patients (13.6%) had stage IIA melanoma, 7 patients (11.9%) had stage IIB melanoma, 5 patients (8.5%) had stage IIC melanoma, 3 patients (5.1%) had stage IIIA melanoma, 5 patients (8.5%) had stage IIIB melanoma, 7 patients (11.9%) had stage IIIC melanoma, and 1 patient (1.7%) had stage IV
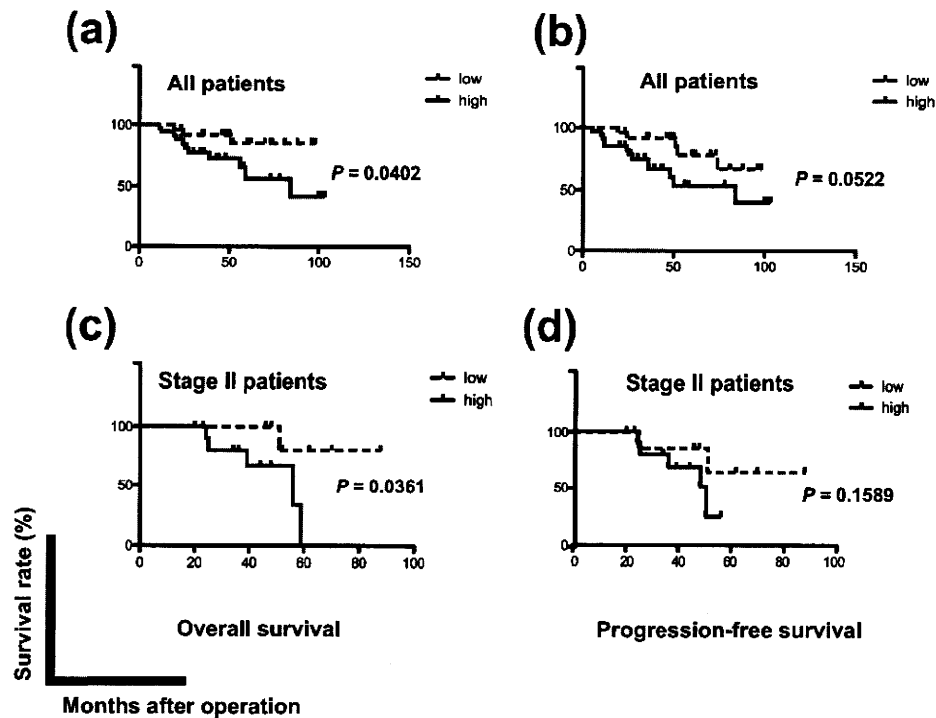
Figure 3. These Kaplan-Meier curves illustrate (a) an analysis of overall survival for patients who had high or low expression of programmed cell death-1 ligand 1 (PD-L1), (b) an analysis of progression-free survival for patients who had high or low expression of PD-L1, (c) an analysis of overall survival for patients with stage II disease who had high or low expression of PD-L1, and (d) an analysis of progression-free survival for patients with stage II disease who had high or low expression of PD-L1.

melanoma. Ulceration was present in 18 patients (30.6%). LN metastases were observed in 16 patients.

PD-L1 Expression and Tumor Cell Expression

The primary tumors from each patient was stained immunohistochemically for PD-L1, and the intensity of its expression level was analyzed as described above (see Materials and Methods). There were 25 patients in the "low-expression" group (RD value, <90) and 34 patients in the "high-expression" group (RD value, ≥90). Representative histopathologic photomicrographs for the high-expression and low-expression groups are provided in Figure 1a and Figure 1b, respectively. The correlations between the PD-L1 expression level and clinical patient profiles are summarized in Table 1. There was no significant correlation between sex and RD or between age and RD. The BTT in the high-expression group was significantly higher than that in the low-expression group ($P = .0298$). The correlation coefficient between the BTT and the RD value was statistically significant (Fig. 2a), indicating that there was a correlation between PD-L1 expression and the vertical growth of malignant melanoma.

Tumors that were classified as T3-T4 exhibited a significantly higher PD-L1 expression than tumors that were classified as T0-T2 ($P = .0072$). PD-L1 expression was not associated with ulceration ($P = .4031$). PD-L1 expression in primary tumors from patients with LN metastasis was significantly higher than that in patients without LN metastasis ($P = .0375$). Furthermore, PD-L1 expression in metastatic LNs was significantly higher than that in nonmetastatic LNs (Fig. 2b) (mean RD value, 128.7 vs 71.4; $P < .0001$). Patients with Clark level IV and V tumors expressed significantly higher RD values than patients with Clark level I and II tumors (Fig. 2c).

Survival and Multivariate Analyses

The overall survival rate was significantly lower in the PD-L1 high-expression group compared with the low-expression group (Fig. 3a, Table 2) according to Kaplan-Meier survival analyses and log-rank tests. The progression-free survival rate tended to differ between the low-expression and high-expression groups (Fig. 3b, Table 2). Among the other clinicopathologic factors, including the clinical melanoma type (superficial spreading melanoma), primary tumor status,

Table 2. Univariate Analysis of Various Factors for Tumor-Specific Death in Patient With Malignant Melanoma

Variable	No. of Patients	OS Univariate RR (95% CI)	P	PFS Univariate RR (95% CI)	P
PD-L1 expression			.0402		.0522
Low	25	1		1	
High	34	3.02 (1.05-8.70)		2.52 (0.99-6.44)	
Age, y			.3399		.3685
≤69	23	1		1	
≥70	36	0.62 (0.23-1.66)		0.64 (0.24-1.70)	
Sex			.3685		.5292
Men	14	1		1	
Women	9	2.44 (0.78-7.65)		1.38 (0.51-3.76)	
Clinical tumor type					
ALM		1		1	
NM		1.20 (0.23-6.37)	.827	0.74 (0.19-2.90)	.6616
SSM		15.94 (2.27-111.8)	.0054	6.03 (1.11-32.71)	.0375
LMM		7.06 (0.24-210.8)	.2596	2.44 (0.16-37.91)	.523
Primary tumor status					
pTis-pT2	24	1		1	
pT3-pT4	35	6.32 (2.01-19.91)		6.66 (1.94-22.89)	
Ulceration					
Absent	41	1		1	
Present	18	3.98 (1.058-15.01)		3.35 (1.11-10.14)	
LN metastases					
pN0	44	1		1	
pN1-pN3	15	22.66 (5.776-88.89)		29.32 (7.18-119.8)	

OS indicates overall survival; PFS, progression-free survival; RR, risk ratio; CI, confidence interval; PD-1, programmed cell death-1 ligand 1; ALM, acral lentiginous melanoma; NM, nodular melanoma; SSM, superficial spreading melanoma; LMM, lentigo maligna melanoma; pT, pathologic tumor classification; pTis, pathologic tumor in situ; pN, pathologic lymph node status.

ulceration, and LN metastasis differed significantly between the 2 expression groups in both overall survival and progression-free survival (Table 2). In patients with stage II melanoma, the high PD-L1 expression group had a significantly lower survival rate than the low-expression group according to log-rank tests (Fig. 3c), and the progression-free survival rate was marginally low without significance in the high-expression group (Fig. 3d). In patients with stage II melanoma, both the low-expression group and the high-expression group exhibited the same BTT levels, but the high-expression group had a significantly lower survival rate. Therefore, we determined that PD-L1 expression is a BTT-independent factor for prognosis.

Multivariate analyses using a Cox proportional hazards model indicated that overall and progression-free survival rates for the high PD-L1 expression group were significantly lower than those for the low-expression group (Table 3). The other factors that contributed to the overall poor survival were primary tumor status, ulceration, and LN metastasis (Table 3), whereas age, sex, and clinical type had no correlation. These data clearly demonstrate that PD-L1 expression in tumor cells is correlated inversely with the prognosis of patients with malignant melanoma and that PD-L1 expression is an independent prognostic factor for both overall and progression-free survival in these patients.

Elevated PD-1 Expression on T Cells in Stage IV Patients

We evaluated PD-1 expression on CD8-positive and CD4-positive T cells in the peripheral blood from patients with stage IV malignant melanoma. Representative flow cytometric data are shown in Figure 4a, which indicates that there was high expression of PD-1 on CD8-positive T cells and on some populations of CD8-negative cells from a patient with melanoma compared with a normal individual. In all patients that we examined who had stage IV melanoma, both CD8-positive and CD4-positive T-cell populations had significantly higher PD-1 levels

Table 3. Cox Multivariate Analysis of Independent Risk Factors for Tumor-Specific Death in Patients With Malignant Melanoma

Variable	No. of Patients	OS Multivariate RR (95% CI)	P	PFS Multivariate RR (95% CI)	P
PD-L1 expression			.0125		.0364
Low	25	1.00		1.00	
High	34	2.04 (1.15-4.26)		1.67 (1.04-2.95)	
Age, y			.904		
≤70	24	1			
≥70	35	0.81 (0.49-1.36)	.403	0.80 (0.51-1.30)	.358
Sex					
Men	38	1.00			
Women	21	1.11 (0.65-1.83)	.685	0.96 (0.57-1.53)	.867
Clinical tumor type					
ALM	39	1.00		1	
SSM	8	1.58 (0.73-2.97)	.2162	1.79 (0.92-3.27)	.0841
NM	10	1.34 (0.63-2.50)	.4133	1.22 (0.57-2.23)	.5696
LMM	2	1.01 (0.00-2.34)	.9868	0.96 (0.23-2.20)	.9404
Primary tumor status					
pTis-pT2	24	1		1	
pT3-pT4	35	4.40 (1.96-18.78)		2.67 (1.52-5.57)	
Ulceration					
Absent	41	1.00		1.00	
Present	18	1.73 (1.04-3.00)		1.84 (1.16-3.05)	
LN metastasis					
pN0	44	1.00		1.00	
pN1-pN3	15	1.69 (1.02-2.80)		1.66 (1.04-2.63)	

OS indicates overall survival; PFS, progression-free survival; RR, risk ratio; CI, confidence interval; PD-1, programmed cell death-1 ligand 1; ALM, acral lentiginous melanoma; SSM, superficial spreading melanoma; NM, nodular melanoma; LMM, lentigo maligna melanoma; pT, pathologic tumor classification; pTis, pathologic tumor in situ; LN, lymph node; pN, pathologic lymph node status.

compared with the levels in normal, healthy controls (Fig. 4b,c). We also examined PD-1 expression levels in tumor-infiltrating, CD8-positive cells in metastatic skin lesions from 2 patients. The tumor-infiltrating, CD8-positive T cells, as represented by the data from 1 patient obtained at the initial occurrence of melanoma and 3 months later (Fig. 5a), revealed increased expression of PD-1 as the disease progressed. The changes in the degree of PD-1 expression on the CD8-positive T cells from these 2 patients are illustrated in Figure 5b.

DISCUSSION

We investigated the expression of PD-L1 in resected specimens from patients with malignant melanoma and observed that there is a correlation between the degree of PD-L1 expression and the vertical growth of primary malignant melanoma. Moreover, our multivariate analysis demonstrated that PD-L1 expression is an independent, poor prognostic factor for malignant melanoma. A representative finding is that the survival rate of the PD-L1 high-expression group was significantly lower than that of the low-expression group with stage II melanoma. Although there has been a report regarding PD-L1 expression on melanoma cells,[19] the clinical significance of PD-L1 in melanoma has not been fully elucidated. Our current study clearly demonstrated the relevance of PD-L1 expression to the growth and prognosis of melanoma cells. The direct involvement of PD-L1 has been demonstrated through the mechanism by which cancer cells escape from the lysis by activated T cells.[28] The expression of PD-Ls on the cell surface of tumor cells, per se, or on antigen-presenting cells in the tumor environment may induce the apoptosis of tumor-reactive T cells through the engagement of PD-1 and, consequently, may promote tumor growth.[19]

Alternative mechanisms underlying the immunosuppression by melanoma cells have been postulated. Patients with melanoma have high serum levels of IL-10,[7] and the number of IL-10–producing monocytes are increased in these patients.[13] It is possible that the elevated
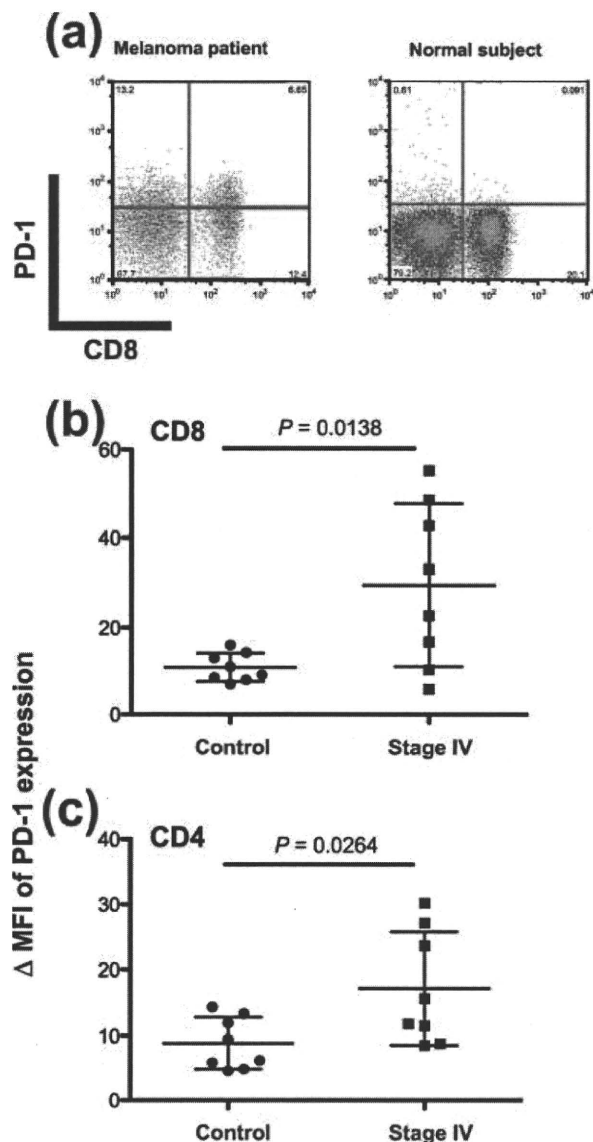
Figure 4. Programmed cell death-1 (PD-1) expression is illustrated in CD8-positive (CD8+) and CD4+ cell populations in peripheral blood. Peripheral blood mononuclear cells were isolated from the peripheral blood of patients with stage IV melanoma and subjected to flow cytometric analysis. Representative flow cytometric analyses of PD-1 expression on CD8+ cells are illustrated in (a) a patient with stage IV melanoma and (b) a normal, healthy control. PD-1 expression is illustrated (a) on CD8+ cells, (b) on CD4+ cells, and in normal, healthy controls. Error bars represent the mean ± standard deviation. MFI indicates mean fluorescence intensity.
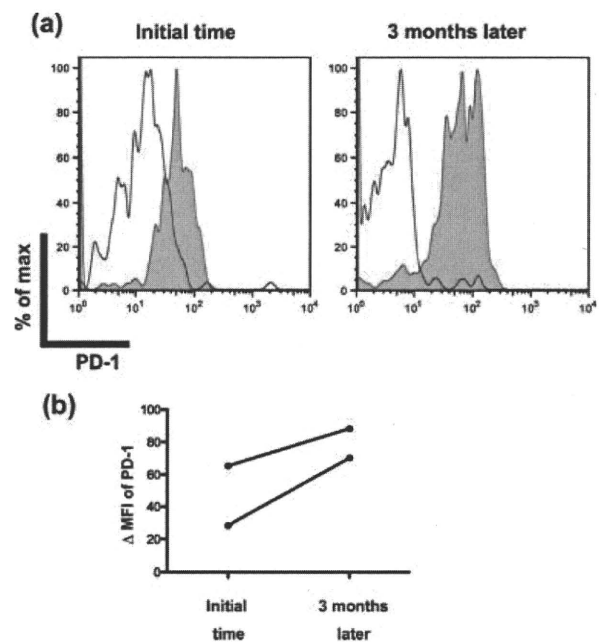

Figure 5. Elevated programmed cell death-1 (PD-1) expression in tumor-infiltrating CD8-positive T cells during tumor progression is shown. (a) PD-1 expression on CD8-positive cells from tumor-infiltrating lymphocytes is illustrated (left) at the initial occurrence of a metastatic skin tumor and (right) 3 months later. The tinted area indicates PD-1 expression; solid line, isotype control. Max indicates maximum. (b) In 2 patients, the alteration of PD-1 expression on CD8-positive cells was monitored. The mean fluorescence intensity (MFI) was calculated as (MFI of PD-1 expression) − (MFI of isotype control).

production of IL-10 by those cells leads to the immunosuppression of tumor immunity by inhibiting cytotoxic T cells or tumor antigen-presenting cells. In addition, IL-10[30] and transforming growth factor-b[31] can be produced by melanoma cells. In another scenario, the melanoma cell also can induce CD25-positive/Foxp3-positive Treg cells.[8] The presence of a high percentage of Treg cells in metastatic LNs also has been reported.[31] Tumor cells spreading into the LN may induce Treg cells, which allow tumor cells to grow locally, and Treg cells may be activated further by unique or shared tumor antigens.[31] A recent finding demonstrated that PD-L1 signaling regulates the conversion of naive, CD4-positive/CD25-negative/Foxp3-negative T cells into FoxP3-positive Treg cells.[32] These findings suggest that PD-L1 on melanoma cells causes immunosuppression by PD-L1–induced Treg cells as well as PD-1/PD-L1 interaction.

PD-1 is expressed on "exhausted" T cells and suppresses immune activation.[33] PD-1 is expressed on post-vaccination, melanoma antigen-specific, cytotoxic T lymphocytes (CTLs).[34] PD-1 blockade during peptide stimulation augmented the absolute numbers of vaccine peptide tetramer-positive CTLs.[34] Our study also demonstrated that PD-1–bearing, CD8-positive cells were increased in PBMCs and in the tumor microenvironment. The number of circulating PD-1–positive/CD8-positive