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In utero manipulation of coat color formation by a monoclonal anti-*c-kit* antibody: two distinct waves of *c-kit*-dependency during melanocyte development

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Previous studies on mice bearing various mutations within the *c-kit* gene, *dominant white spotting* (*W*), indicate the functional role of this tyrosine kinase receptor in the development of melanocytes, germ cells and hematopoietic cells. Despite the availability of mice defective in the *c-kit* gene and a respectable understanding of the molecular nature of *c-kit*, however, it is not clear at what stage of gestation *c-kit* is functionally required for the development of each of these cell lineages. To address this question, we have used a monoclonal anti-*c-kit* antibody, ACK2, as an antagonistic blocker of *c-kit* function to interfere with the development of melanocytes during embryonic and postnatal life. ACK2 injected intradermally into pregnant mice entered the embryos where it blocked the proper development of melanocytes. This inhibitory effect was manifested as coat color alteration in the offspring. Furthermore, ACK2 injection also altered the coat color of neonatal and adult mice. Based on the coat color patterns produced by ACK2 administration at various stages before or after birth, the following conclusions are drawn: (i) during mid-gestation, *c-kit* is functionally required during a restricted period around day 14.5 *post-coitum* when a sequence of events leading to melanocyte entry into the epidermal layer occurs; (ii) during postnatal life, *c-kit* is required for melanocyte activation which occurs concomitantly with the hair cycle which continues throughout life after neonatal development of the first hair.

Key words: coat color alteration/hair follicle/*c-kit*/melanocyte development/monoclonal anti-*c-kit* antibody

Introduction

c-kit is a gene encoding a tyrosine kinase receptor, in the PDGF receptor/CSF-1 receptor family (Besmer *et al.*, 1986; Yarden *et al.*, 1987; Qiu *et al.*, 1988). Recently, *c-kit* was mapped to the *dominant white spotting* (*W*) locus (Chabot *et al.*, 1988; Geissler *et al.*, 1988), whose phenotype has been extensively analyzed for more than two decades (reviewed in Russell, 1979; Silvers, 1979; Green, 1981). Moreover, cDNA of the ligand for murine *c-kit*, SCF/KL/MGF, has been recently cloned (Zsebo *et al.*, 1990; Huang *et al.*, 1990; Anderson *et al.*, 1990) and mapped to the *Steel* (*Sl*) locus (Copeland *et al.*, 1990; Zsebo *et al.*, 1990; Huang *et al.*, 1990), whose phenotype is basically identical to *W* (Mayer, 1970; Silvers, 1979; Russell, 1970; Kitamura and Go, 1979). According to previous phenotype analysis of *W* and *Sl* mice, it is clear that *c-kit* and its ligand play a crucial role in the development of hematopoietic cells, germ cells and melanocytes. Thus, study of the functional role of *c-kit* and its ligand has already reached the level where cloned genes, considerable understanding of the molecular nature of the cloned genes, and even mice with mutated genes are available. This level has in fact been one of the goals of recent developmental biology, as seen in the recent extensive attempts to produce a model mouse which is defective for a specific gene. Nevertheless, since this level has now been attained for *c-kit* and its ligand, the question to be addressed is whether previous phenotype analysis of *W* or *Sl* mice has revealed every *c-kit*-dependent process in the differentiation of each cell lineage.

Phenotypic analysis of *W* and *Sl* mice has unequivocally proven the involvement of *c-kit* in melanocyte development in embryonal and postnatal life. However, previous studies have not specified at which stage of melanocyte development *c-kit* is functionally required. Even the most recent papers studying the expression of *c-kit* and SCF/KL/MGF genes could go no further than mentioning that *c-kit* is involved in the migration of melanocyte precursors into hair follicles (Orr-Urtreger *et al.*, 1990; Matsui *et al.*, 1990). Major limitations of previous phenotype analyses have been as follows. Firstly, there are no good markers which discriminate melanocyte precursors from surrounding cells during their migration from the neural crest to the hair follicle. Thus, early defects in melanocyte development in *W* or *Sl* mice are difficult to characterize in tissues. Secondly, since developmental failure of melanocytes results in a hair follicle deficient in melanocytes, it is difficult to investigate the role of *c-kit* in melanocyte activation in postnatal life. Thus, markers for melanocyte precursors and methods of controlling the function of *c-kit* at a given time of pre- and postnatal life *in vivo* will be particularly important for further investigation.

In the present study, we demonstrate that an antagonistic monoclonal antibody can meet both these demands, as not only a marker for *c-kit*$^+$ cells but also an antagonistic blocker capable of controlling *c-kit* function *in vivo*. By using this antibody, we identify at least two distinct *c-kit*-dependent processes during melanocyte development, one during the proliferation of melanocyte precursors in the mesodermal layer, which eventually leads to the melanocyte entry into the epidermal layer, and the other during melanocyte activation along with the hair cycle in postnatal life.

Results

Preparation of rat anti-murine-c-kit monoclonal antibody

Since it has been shown that the *W/W* mouse cannot express *c-kit* due to a mutation at a splice donor site which results in deletion of exons including the transmembrane domain (Nocka *et al.*, 1990, Hayashi *et al.*, 1991), hybridomas were produced from the spleens of rats which had been immunized with IL-3-dependent mast cell lines from normal mice, and screened for antibodies which bind to IL-3-dependent mast cells from normal mice but not to those from *W/W* mice. Four such hybridomas were produced, all of which stained COS7 cells transfected with the *c-kit* gene in an expression vector but not COS7 cells transfected with an insert-free vector (data not shown). Among these, ACK2 completely suppressed hematopoiesis in the normal mouse (Ogawa *et al.*, 1991) and was therefore chosen for this study.

Coat color alteration in offspring by ACK2 injection into pregnant mice

It is well established that rat IgG2b injected into pregnant mice is transferred to embryos through the placenta (Kimoto and Kishimoto, 1986; Zuniga-Pflucker *et al.*, 1989). Thus, it may be expected that *c-kit*-dependent processes in the embryo can be controlled by an injection of the antagonistic anti-*c-kit* antibody, ACK2 into the pregnant mouse. With regard to melanocyte development, therefore, the effect of ACK2 administration would be manifested as coat color alterations of the offspring.

In preliminary experiments, various doses of ACK2 were injected by various routes into pregnant C57BL/6 (B6) mice at varying days of gestation, from days 10.5 to 16.5 *post-coitum* (p.c.) and the coat color of the offspring was examined at day 14 after birth. These preliminary experiments were continued until we found a method which produced unpigmented offspring. Eventually the intradermal injection of 2 mg ACK2 was found to be the most suitable method. It is important to note that 1 mg ACK2 by the same route had no effect on coat color. On the other hand, the offspring from mice given an intradermal injection of 3 mg ACK2 were born alive but died within 24 h of birth. Some of these offspring were examined histologically and were found to have no hematopoietic cells in the bone marrow (data not shown). In fact, we have demonstrated that ACK2 injection even into the adult mouse blocked the proliferation of hematopoietic stem cells, which eventually resulted in depletion of the hematopoietic cells from the bone marrow (Ogawa *et al.*, 1991). Thus, consistent with the previous phenotype analyses of *W* mice (Russell, 1979), the proliferation of hematopoietic stem cells in embryos is dependent on *c-kit*.

After the dose and route of ACK2 injection had been fixed at 2 mg intradermal injection, we reproducibly obtained unpigmented offspring from B6 mice, and they survived to adulthood. However, the timing of injection which could induce the coat color alteration was restricted. Injection at day 10.5 p.c. or day 11.5 p.c. had basically no effect on coat color, although 5 of 34 offspring of this group had small, asymmetrical unpigmented islands in the ventral region and unpigmented hindlimbs (Figure 1). Injection later than day 15.5 p.c. had no effect on the coat color of offspring (Figure 2B). We could not evaluate the effect of 2 mg ACK2 on the coat color formation of day 12.5 p.c. embryos due to perinatal death of the offspring by aplastic anemia. By the injection at day 13.5 p.c., the entire coat of the offspring was unpigmented, except the vibrissae and the auricle (Figure 2A). Surprisingly, an injection at day 14.5 p.c. caused strikingly variable coat color patterns. The mice shown in Figure 2D–G are litter-mates from a B6 mouse and represent four typical patterns of pigmentation produced repeatedly by the same treatment (see also Figure 3). The first pattern (Figure 2D) is the same as that seen in the offspring from the day 13.5 p.c. injected mice. The second pattern shows sparsely pigmented areas in the mid-dorsal trunk and also pigmented spots on the face (Figure 2E). In the third pattern, the sparsely pigmented areas observed in the second pattern become fully pigmented, and the pigmentation extends (Figure 2F). The final pattern has mostly pigmented coat except midline of the ventral region and the face (Figure 2G). It is important to note that the coat color alteration induced by ACK2 injection during embryonal life is permanent. Figure 3 shows the coat color patterns of two-month-

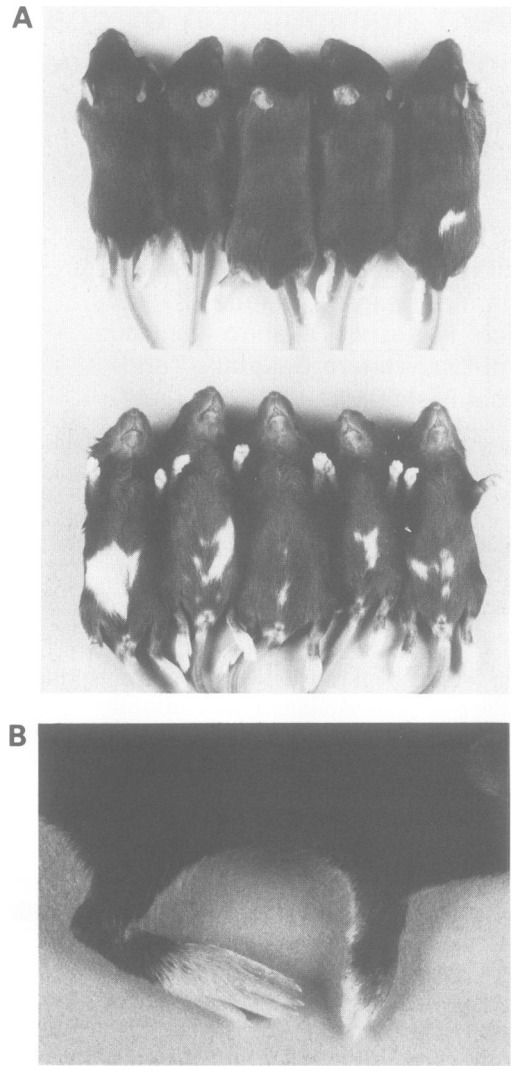

Fig. 1. Coat color patterns of offspring from a mother ACK2 treated at day 10.5 p.c. Five pregnant C57BL/6 mice were given an intradermal injection of 2 mg ACK2 at day 10.5 p.c.. 34 offspring survived to adulthood, and five showed unpigmented spots and unpigmented hind-limbs.

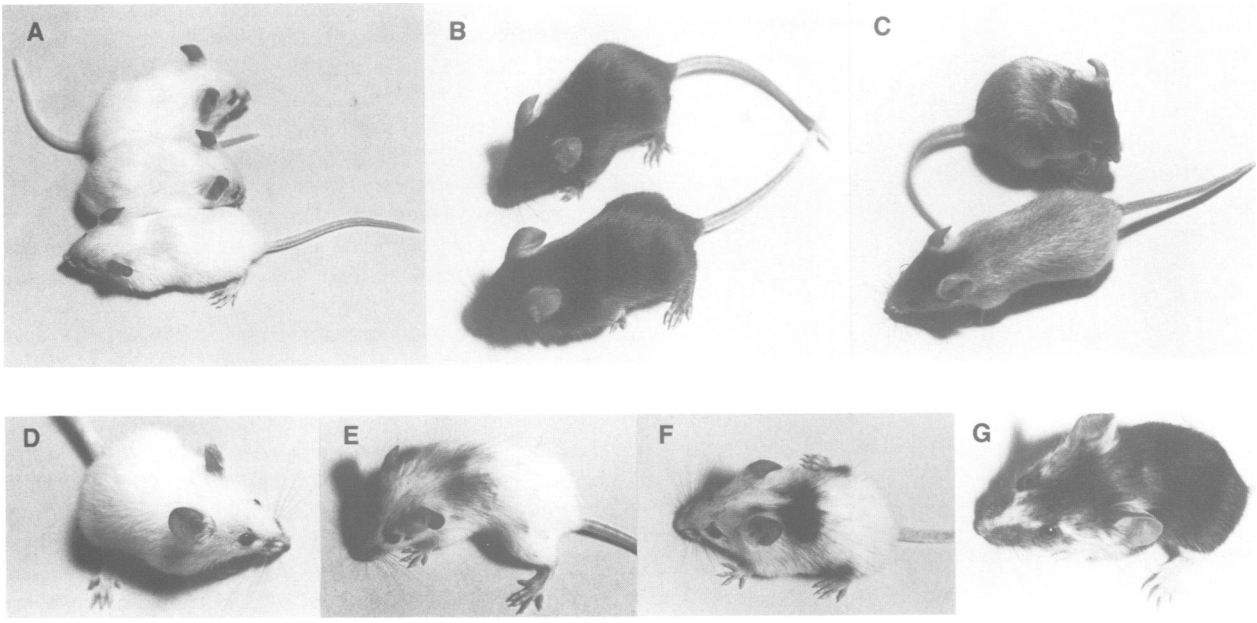

Fig. 2. Effect of ACK2 on the coat color formation of mid-gestational embryo. Pregnant C57BL/6 mice were given a single intradermal injection of 2 mg ACK2 at various days *post-coitum*. For neonates, intraperitoneal injection of 50 μg ACK2 was given twice at days 3 and 6 post-partum. (**A**) day 13.5 p.c. treated offsprings, (**B**) day 15.5 p.c. treated offspring (upper mouse) and day 16.5 p.c. treated offspring (lower mouse), (**C**) neonatally treated mice, and (**D**–**G**) day 14.5 p.c. treated litter-mates.

old litter-mates from a B6 mouse given an ACK2 injection at day 14.5 p.c., demonstrating the stability of the thus produced coat color for 2 months after birth. The same dose of anti-CD4 (GK1.5) used as a class-matched non-binding control antibody had no effect on coat color (data not shown).

Expression of c-kit in the cutaneous tissue of embryos

In the preceding section, we were able to specify a process which occurs at around day 14.5 p.c. during melanocyte development as *c-kit*-dependent. What known events of melanocyte development correspond to this process? Since a previous histological study reported that the first invagination of the epidermal layer leading to the formation of hair follicles starts at day 14 p.c. (Rugh 1968), we suspected that it is sequence of events leading to the melanocyte colonization of the developing hair follicle which is *c-kit*-dependent. To test this possibility, we prepared the cranio-facial skin of day 12.5 p.c. embryos and the dorso-lateral skin of day 14.5 p.c. and day 15.5 p.c. embryos and examined the expression of *c-kit* immunohistochemically (Figure 4). Only few, if any, *c-kit*$^+$ cells were present in the skin of day 12.5 p.c. embryos. In the mesodermal layer of the day 14.5 p.c. embryo, however, a significnat number of *c-kit*$^+$ cells were present; this number increased on the following day. Interestingly, *c-kit*$^+$ cells in the epidermal layer of the skin were first detected at day 15.5 p.c. Taken together the entry of *c-kit*$^+$ cells into the epidermal layer appears to take place from day 14.5 p.c. to day 15.5 p.c. Since ACK2 injection at 15.5 p.c. has no effect on the coat color, the distribution of the precursor cells which function as stem cells for melanocytes in hair follicle must be complete by about this stage. Therefore, a sequence of events leading to melanocyte-entry in epidermal layer is most likely to be the process which is *c-kit*-dependent.

Accessibility of maternally administered ACK2 to the cutaneous tissue of embryo

To interpret our results from the preceding sections properly, there remain questions to be resolved. Most important is the penetration of ACK2 into the embryo. In a previous study, it was demonstrated that maternally-injected monoclonal antibody, even though it recognized H-2 antigen on the placental cells, was transmitted to the embryo as early as day 10 p.c. (Raghupathy *et al.*, 1981). Furthermore, we measured the absolute amount of ACK2 in maternal and embryonal blood by enzyme linked immunosorbent assay (ELISA), and found that ACK2 did enter the embryo. It is important to note, however, that the concentration of free ACK2 in the embryonic blood was 500–1000 times less than that present in maternal blood, and the maximum concentration of free ACK2 in the embryo under our experimental condition (intradermal injection of 2 mg ACK2) was within the range 200–400 ng/ml. Whether this value has any significant meaning or not is difficult to conclude, because we expect that most of the antibody is trapped by *c-kit*$^+$ cells. Furthermore, a large enough blood sample can only be collected from the embryo after day 14.5 p.c. Thus, one might argue that the pigmented area of ACK2 treated offspring is where the antibody was inaccessible. In order to exclude this possibility, we attempted to demonstrate the localization of ACK2 in the embryo immunohistochemically. The embryos examined were from day 10.5 p.c. or day 15.5 p.c. treated mice, because ACK2 treatment at these stages failed to alter the coat color (Figures 1 and 2). Relatively thick (10 μm) frozen sections of embryos from ACK2 treated mice were prepared, and the localization of ACK2 was determined immunohistochemically by using anti-rat IgG. Although free ACK2 in loose tissues was easily washed off during the staining procedure, *c-kit*$^+$ cells and also the dermal layer which is

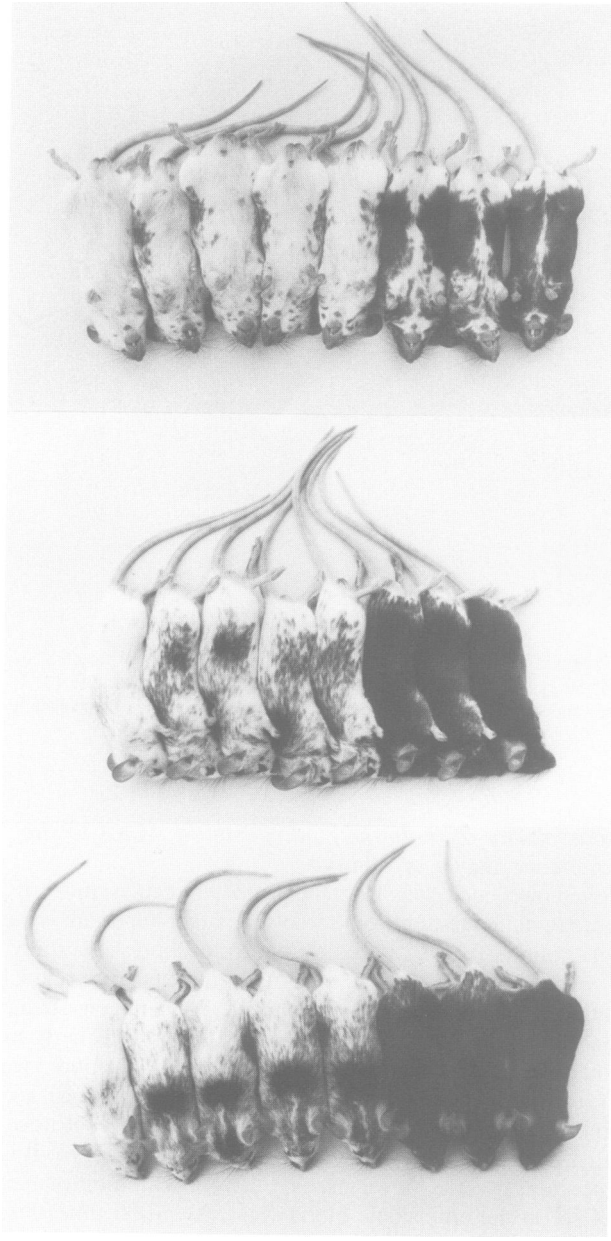

Fig. 3. Coat color patterns of litter-mates from a mother treated with 2 mg ACK2 at day 14.5 p.c. A pregnant C57BL/6 mouse was given an intradermal injection of 2 mg ACK2 at day 14.5 p.c. Eight litter-mates were obtained, and the coat color pattern was examined 2 months after birth.

relatively dense with extracellular matrix could retain ACK2. ACK2 injected intradermally into pregnant mice at day 10.5 p.c. was detected in the dermal layer of the embryos 24 h but not 8 h after the injection (Figure 5). The concentrations of ACK2 in the maternal blood were 53.2, 187.1 and 64.0 μg/ml at 8, 24 and 48 h after the injection respectively. This suggests that ACK2 did enter the embryos and reached the dermis within 24 h after the injection into maternal skin. A more striking histological picture was obtained when an intradermal injection of ACK2 was given to day 15.5 p.c. mice and the cutaneous tissue of dorsal region of the embryo was prepared 24 h later. In this experiment, ACK2 concentrations in maternal and embryonic blood were 165 μg/ml and 248 ng/ml respectively. As seen in Figure 5E, the cells which trapped injected ACK2 were present both in mesodermal and epidermal layers. These ACK2 bearing cells are likely to correspond to those identified as *c-kit*$^+$ cells in Figure 4. It is important to note that ACK2 injected into day 15.5 p.c. mice bound to *c-kit*$^+$ cells in the epidermal layer, although it has no effect on the coat color of offspring. This indicates that melanocytes in the epidermal layer, although accessible to ACK2, do not require *c-kit* for their survival, and therefore ACK2 injection at this stage failed to alter the coat color of offspring.

ACK2 blocks the increase of c-kit$^+$ *cells in the cutaneous tissue of the embryo*

Our immunohistological study on the cutaneous tissue of normal embryos demonstrated that *c-kit*$^+$ cells in the mesodermal layer increased from day 12 p.c. to day 14 p.c. before entering into the epidermal layer. In this section, we determined whether this increase is blocked by ACK2 injection. Pregnant mice were given an intradermal injection of 2 mg ACK2 at day 13.5 p.c., and 2 days later the cutaneous tissue of the dorsal region of the embryo was stained with ACK2 (Figure 6). In the tissue of control mice, *c-kit*$^+$ cells were present both in the mesodermal and epidermal layers. On the other hand, no *c-kit*$^+$ cells were detected in the tissue from ACK2 injected mice, although the dermis showed higher background due to the retained ACK2. This result indicates that the increase of *c-kit*$^+$ cells in the mesodermal layer is blocked by ACK2 injection, which eventually results in the hair follicle being depleted of melanocytes.

c-kit dependency of melanocyte activation in postnatal life

Finally, we investigated whether or not ACK2 injection in postnatal life is also effective in altering the coat color of mice. First, 50 μg ACK2 was injected into neonates at days 3 and 6 *post-partum* (p.p.), and their coat color was examined at day 14 p.p. As shown in Figure 2C, the dilution of the entire coat was induced by this treatment. When ACK2 injection started on the day of birth, more intensive coat color dilution was produced (data not shown). An intradermal injection of 2 mg ACK2 also affected the coat color of adult mice (Figure 7A). In contrast to neonatal injection, however, only a small part of the coat became unpigmented. Importantly, these unpigmented areas showed no specific correlation with the ACK2 injected site and appeared more than a month after the injection. Moreover, location of the unpigmented areas induced by this treatment varied among mice. Thus, we surmise that these unpigmented areas may correspond to the areas in which the hair cycle was activated at the time of ACK2 injection. In order to test this possibility, we artificially activated the hair cycle by shaving the hair, and injected 2 mg ACK2 on the next day. Figure 7B showed that the regenerated hair of the shaved area was unpigmented. Again in this mouse, ACK2 injected site remained pigmented. This result clearly indicates that ACK2 enters the fully developed hair follicle and blocks the activation of melanocytes at the initial phase of the hair cycle.

Discussion

Our study demonstrated that ACK2, a monoclonal antibody against *c-kit*, when injected into pregnant mice enters the embryo and causes irreversible blockage of the development

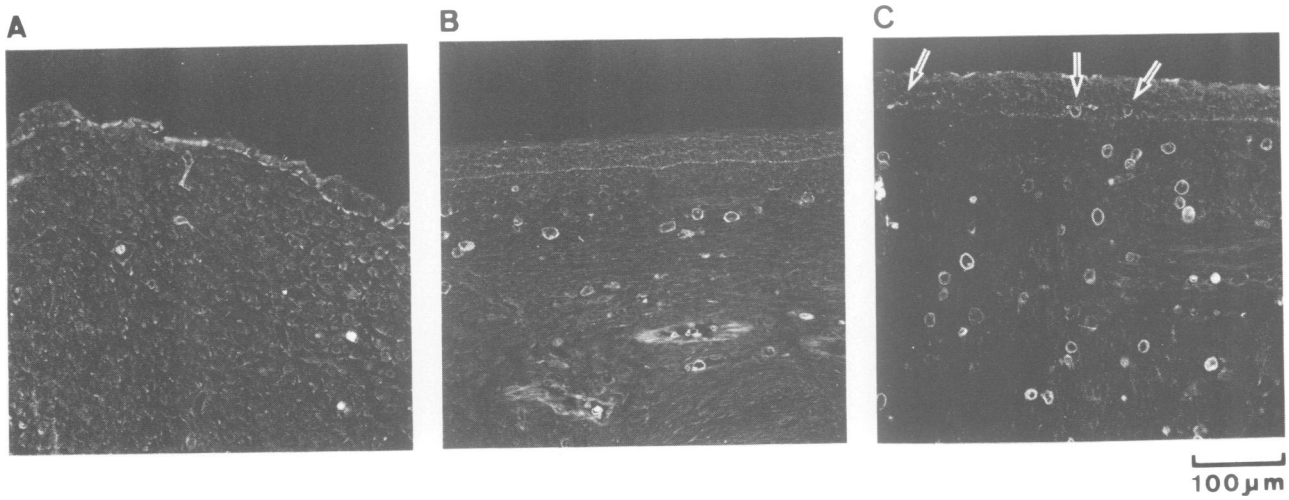

Fig. 4. ACK2 staining of dermal tissues of embryos. Skin of embryo was prepared and stained with ACK2. (**A**) day 12.5 p.c., few *c-kit*$^+$ cells are seen underneath the epidermis, (**B**) day 14.5 p.c., numerous *c-kit*$^+$ cells. (**C**) day 15.5 p.c., *c-kit*$^+$ cells are noticed in the epidermal layer (arrows).

of melanocytes. In addition, the fact that the offspring that were given higher doses of ACK2 died of aplastic anemia suggests that the development of the hematopoietic system is blocked by a single injection of the same antibody. Interestingly, however, all the offspring obtained from ACK2 injected mice, which survived to adulthood, were fertile irrespective of their coat color (data not shown), suggesting that one injection at these embryonal stages is not sufficient for suppressing the germ cell development. Thus, although previous phenotypic analysis of *W* mutant mice clearly demonstrated the role of *c-kit* in the development of these cell lineages, the timing and mode of *c-kit* function may differ with cell lineages. The roles of *c-kit* in the development of hematopoietic cells and germ cells are currently under investigation. Nevertheless, it is likely that monoclonal antibodies recognizing the active site of any membrane molecules can be utilized to investigate their functional roles at a given stage during embryogenesis. In fact, this strategy has been successfully used to manipulate the process of T lymphocyte development in embryos (Kimoto and Kishimoto, 1986; Zuniga-Pflucker *et al.*, 1989). There are many receptor molecules whose roles in embryogenesis remain unknown. We expect that antibody-mediated manipulation will provide a useful tool for this purpose.

There are two possible mechanisms for the action of the antibody administered to embryo. One is by antibody-mediated cytotoxic reactions, and the other is by antibody-mediated receptor blockage. In the former case, all *c-kit*$^+$ cells are depleted, whereas in the latter case, *c-kit*-dependent signal transduction is blocked. Although it is difficult to exclude the former completely, we think the latter possibility more likely for the following reasons. First, despite the high expression of *c-kit* in the placenta and the peripheral nervous plexus in the intestine (M.Kusakabe, unpublished observation), ACK2 injection had little, if any, effect on the development of these tissues. Secondly, although melanocytes in the epidermis of day 15.5 p.c. embryo are *c-kit*$^+$, ACK2 injection at this stage did not alter coat color formation. If ACK2 is cytotoxic to *c-kit*$^+$ cells, the coat color of day 15.5 p.c. treated embryos must be unpigmented. Finally, a previous study using GK1.5, also rat IgG2b, clearly demonstrated that the major action of GK1.5 on the developing thymus is cytostatic rather than cytotoxic (Zuniga-Pflucker *et al.*, 1989).

Given that ACK2 blocks the function of *c-kit*, the question to be addressed is whether the experiments using ACK2 could increase our understanding of the functional role of *c-kit* beyond what had been accumulated by previous phenotype analysis of *W* or *Sl* mouse. We consider that the present results do provide novel information. First, although it is well established that *c-kit* is involved in the migration of melanoblasts from the neural crest (Mayer, 1970; Silvers, 1979), previous phenotypic analyses of *W* and *Sl* mice could not specify where and when *c-kit* is actually required. On the other hand, ACK2, which is capable of marking *c-kit*$^+$ cells and of blocking the function of *c-kit*, enabled us to specify the sequence of melanocyte development at around day 14.5 p.c. as being *c-kit*-dependent. Moreover, our immunohistological examination demonstrated that this process includes the proliferation of melanoblasts in the mesodermal layer and their entry into the epidermal layer, and also that ACK2 injection blocks the increase of *c-kit*$^+$ cells in the mesodermal layer. It is important to note the finding of Matsui *et al.* (1990) that the ligand for *c-kit* is expressed in the mesodermal, but not the epidermal layers, suggesting that *c-kit* and its ligand are not directly involved in melanoblast colonization. In fact, our results showed that although *c-kit*$^+$ cells were present in the epidermal layer of day 15.5 p.c. embyro, ACK2 injection at this stage had no effect on coat color formation. This suggests that the melanocytes became quiescent upon entering into the epidermal layer due to the absence of the ligand and were therefore not affected by ACK2 injection. Taken together, it is likely that *c-kit* and its ligand are required for the melanoblast proliferation in the mesodermal layer, and that the molecule(s) which guide melanoblasts into the epidermal layer are induced during this proliferation phase. Previous observation that a large number of differential melanocytes persisted in the mesodermal layer of normal neonates but not of *Sl*/*Sl*^{d} neonates also suggests that *c-kit*-dependent proliferation of melanoblasts occurs in the mesodermal layer (Nakayama *et al.*, 1988).

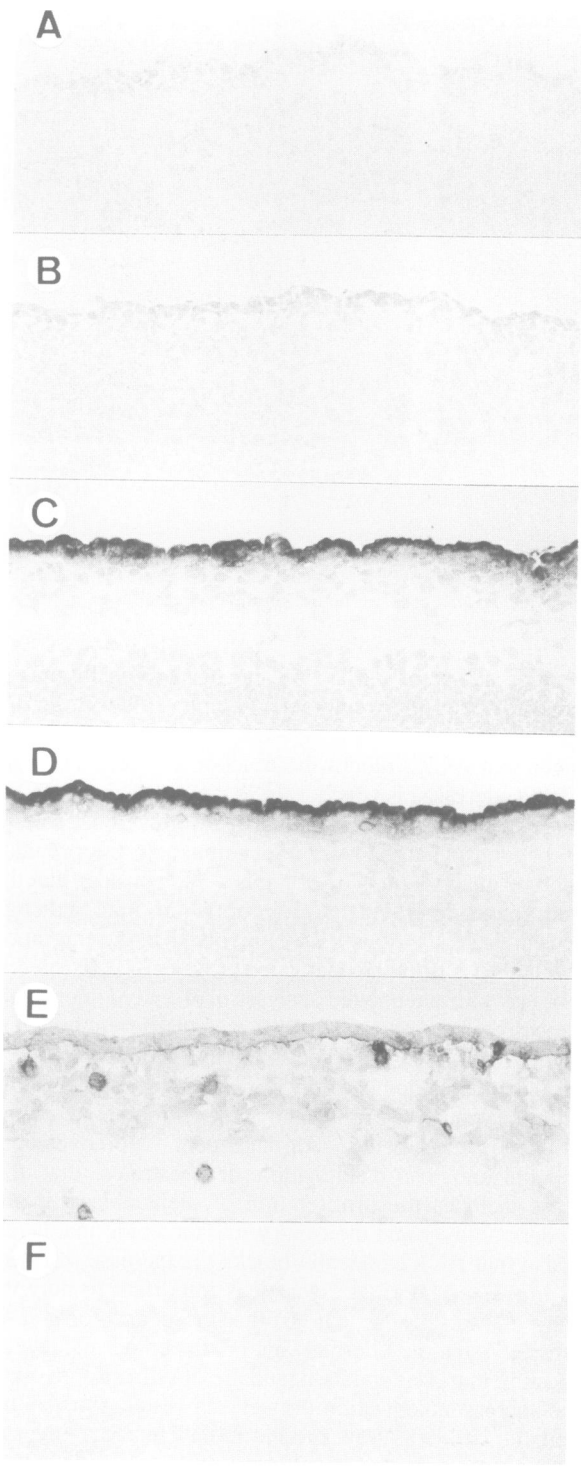

Fig. 5. Immunohistochemical detection of maternally injected ACK2 in the cutaneous tissue of the embryos. Pregnant mice were given 2 mg of ACK2 at day 10.5 or 15.5 p.c. After varying times of incubation, the embryo was removed, 10 μm frozen sections were prepared, dried, fixed with 95% ethanol−1% acetic acid, and stained directly with biotin-labeled anti-rat IgG. Subsequent procedure was as recommended in the manual of Zymed streptavidin−biotin immunohistological staining kit. (**A**) 0 h, (**B**) 8 h, (**C**) 24 h. (**D**) 48 h after ACK2 injection into a day 10.5 p.c. mouse, ACK2 retained in the dense matrix is clearly visible 24 h after the injection. (**E**) 24 h after the injection into a day 15.5 p.c. mouse, ACK2 trapped on the surface of *c-kit*$^+$ cells are detectable by anti-rat IgG, and ACK2-bearing cells are present both in the mesodermal and epidermal layers. (**F**) A control 12.5 day p.c. embryo from a non-treated mouse. The skins of dorsal region were shown.

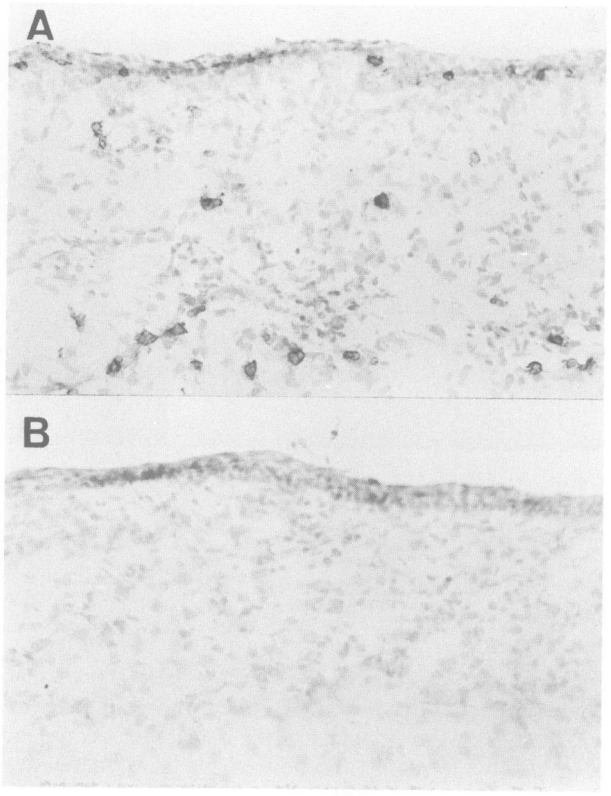

Fig. 6. ACK2 staining of dermal tissues of the embryos from the mouse injected with ACK2 at day 13.5 p.c. A pregnant mouse was given 2 mg intradermal injection of ACK2 at day 13.5 p.c., and the embryos were recovered 2 days later. A sagittal section of whole embryo from (**A**) a normal mouse or (**B**) an ACK2 treated mouse was prepared and stained with ACK2 and Zymed streptavidin−biotin immunohistological staining kit. The skins of dorsal regions are shown. *c-kit*$^+$ cells which are present in the dermis of the control embryo (A) are not found in the dermis of the ACK2 treated embryo (B).

Secondly, the coat color patterns shown in Figure 3 are entirely new. Since these patterns were produced by injecting ACK2 at day 14.5 p.c., the unpigmented areas represent regions where the *c-kit*-dependent process is in progress when the effective concentration of ACK2 is sufficiently high in the embryo. In the pigmented areas, this process would have already been completed before ACK2 reached an effective dose. The striking variation of the coat color pattern in litter-mates from a day 14.5 p.c. treated mouse indicates that this process is completed within a short period of time of the order of the difference in the gestational stage among litter-mates, that is a few hours only. Given that this process is a sequence of events leading to melanocyte colonization of the epidermal layer, the pigmentation patterns of these litter-mates dynamically demonstrate the part of the coat from which colonization starts, the order in which it proceeds, and where it finishes.

Finally, the present results demonstrate that *c-kit* is required for the activation of melanocytes in the fully developed hair follicles in postnatal life. This conclusion cannot be drawn from phenotypic analysis of mutant mice, although it could have been anticipated, because the hair follicles of mutant mice are depleted of melanocytes. We also demonstrated that this activation is regulated in coordination with the hair cycle. Previous histological analysis

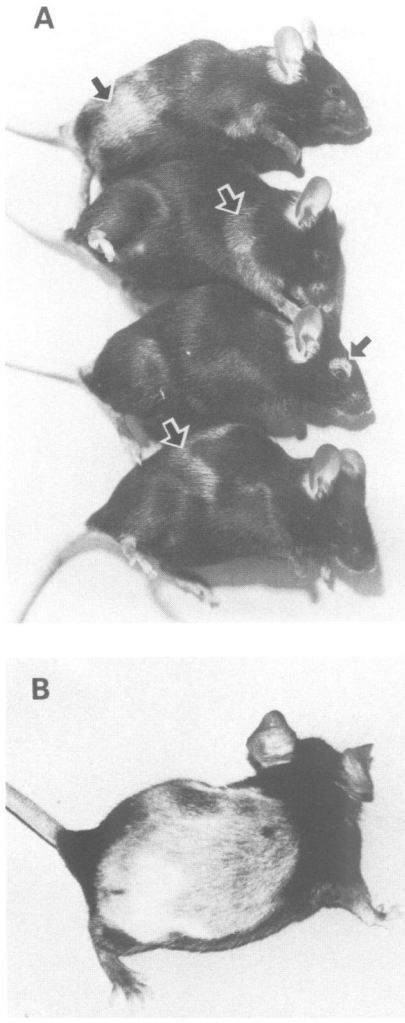

Fig. 7. Effect of ACK2 injection on the coat color of adult mouse. (**A**) 10 week-old C57BL/6 mice were given an intradermal injection of 2 mg ACK2 and coat color was examined 6 weeks later. Arrows indicate the unpigmented areas which appeared in these mice. (**B**) Dorsal hair of a 10 week-old C57BL/6 mouse was shaved using an electric shaver and 2 mg ACK2 was injected intradermally the following day. The coat color was examined 8 weeks after the injection.

indicated that most melanocytes disappear at the terminal phase of the hair cycle, thus melanocyte stem cells must be activated for proliferation at the initial phase of the next cycle (Silvers 1979). Consequently, *c-kit* would be required for melanocyte proliferation at the initial phase of the hair cycle. How the activation of hair cycle can trigger *c-kit*-dependent proliferation of melanocytes remains to be elucidated. However, as the ligand for *c-kit* as well as antagonistic anti-*c-kit* antibody are now available, this question should be answered in the near future.

In the present study, we identified two *c-kit*-dependent processes in melanocyte development. One is a sequence of events leading to melanocyte colonization of the epidermal layer at around day 14.5 p.c., and the other is melanocyte activation in developed hair follicles concomitant with the hair cycle. However, this study cannot conclude that there are no other *c-kit*-dependent processes during melanocyte development. Obviously, an earlier process before the development of a functional placenta could not be examined using the present strategy. Furthermore, the fact that ACK2 injection at day 10.5 p.c. produced, although only occasionally, unpigmented spots suggests the presence of another *c-kit*-dependent process. However, since this phenotype presently occurs only sporadically, the type of process which causes these asymmetrical unpigmented spots requires further investigation. Nevertheless, when other appropriate techniques are combined with the ACK2 injection described here, study of the earlier process of melanocyte development will become feasible.

Materials and methods

Mice and convention for timing of pregnancy

C57BL/6 and WB-*W*/+ mice were purchased from Shizuoka Agricultural Co-operation for Laboratory Animals (Shizuoka, Japan). *W*/*W* mice were obtained by the cross with WB-*W*/+ parents. Female C57BL/6 mice were mated with males at around 6 p.m. At 9 a.m. the next morning, mice with a vaginal plug were separated. Assuming that fertilization takes place around midnight, noon on the day on which the vaginal plug is found is scored as day 0.5 p.c.

Production of monoclonal antibody and preparation of purified antibody

The hybridoma was produced as previously described (Nishikawa *et al.*, 1986). ACK2 was produced from the fusion with the spleen from a rat which had been immunized twice with 2×10^7 of IL-3-dependent mast cell lines. Hybridomas secreting the antibody which bound to mast cell lines from normal mice but not those from *c-kit*-defective *W*/*W* mice were selected, cloned twice and expanded. Specificity of the antibodies was finally determined by using COS7 cells transfected with *c-kit* cDNA cloned into CDM8 expression vector (Aruffo and Seed 1987), using COS7 cells transfected with CDM8 alone as negative controls. Among four anti-*c-kit* antibodies established, ACK2 showed the strongest inhibition of the hemopoiesis in the Dexter's long-term bone marrow culture (Dexter *et al.*, 1977; Nishikawa *et al.* 1988), and also suppressed the myelopoiesis and erythropoiesis in adult bone marrow (Ogawa *et al.*, 1991). This antibody was therefore selected for this study. For purification of antibody, we cultured ACK2 in AFS104 serum free medium (Ajinomoto, Tokyo). After incubation, the culture supernatant was precipitated with saturated ammonium sulfate at 50% (v/v) concentration, and the precipitate was further subjected to DE52 (Whatman) column chromatography. This procedure provides an almost pure antibody preparation. As a class-matched control monoclonal antibody, we used purified anti-CD4 (GK1,5; Wilde *et al.*, 1983) which does not bind to melanocytes. As injection of this antibody had no effect on coat color of embryos, the results are now shown.

Mast cell lines

IL-3-dependent mast cell lines were established from bone marrow cells of normal or *W*/*W* mouse, essentially as described by Nakano *et al.* (1985). In this study, we used murine recombinant IL-3 (Hattori *et al.*, 1987) instead of conditioned medium of spleen cells stimulated by pokeweed mitogen. By repeating the subculture weekly in RPMI 1640 (Gibco) containing 10% calf serum (Hyclone) and 5×10^{-5} 2-mercaptoethanol, a pure mast cell population was usually obtained after 4−5 weeks. To establish mast cell lines from a *W*/*W* mouse, we used new-born mouse bone marrow cells as the starting cell population.

Immunohistochemistry

Embryos *in utero* were placed in 0.1 M sodium phosphate buffer (pH 7.4) and irradiated using a microwave oven (BioRad H25000) for 20 min at 60% power level at 40°C as prefixation. The embryos were then post-fixed in ice-cold 95% ethanol containing 1% acetic acid, dehydrated with 100% ethanol, embedded in polyester wax and sectioned at 4 μm. Sections were rinsed in 100% ethanol to remove the wax and subsequently ethanol was washed out with PBS. These sections were pre-incubated with PBS containing 5% normal goat serum and 1% BSA to block non-specific antibody reactions and then incubated with anti-*c-kit* for 1 h at room temperature. After washing, sections were incubated with FITC-labeled anti-rat IgG for 1 h at room temperature, washed and examined by fluorescent microscopy. In one experiment, ACK2 incubated sections were developed using a streptavidin-biotin-peroxidase staining kit (Zymed Laboratories, CA). For demonstrating the tissue localization of ACK2, frozen sections of the embryos

from ACK2 injected mice were prepared, dried, fixed with ethanol–acetic acid, and stained directly with biotin-labeled anti-rat IgG using a streptavidin–biotin staining kit (Zymed Laboratories).

Determination of ACK2 concentration by microwell ELISA

A 96 well microtitre plate was coated with 10 μg/ml anti-rat IgG2b monoclonal antibody (MARG2b-8, Zymed) and blocked with 1% BSA–PBS. The wells were incubated with various dilutions of serum samples, washed with PBS, and incubated with horseradish-peroxidase labeled anti-rat-κ monoclonal antibody (Zymed). After washing with PBS, 100 μl of *o*-phenylendiamine in citrate buffer was added, and absorption at 490 nm was measured 30 min later. The absolute concentration of ACK2 was calculated from the standard curve of purified ACK2.

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