Bcl-2 Reduced and Fas Activated by the Inhibition of Stem Cell Factor/KIT Signaling in Murine Melanocyte Precursors

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Stem cell factor (SCF) and its receptor, KIT, are essential to the migration and differentiation of melanocytes during embryogenesis. We previously demonstrated that apoptosis is induced by blocking survival function of the SCF/KIT interaction in a mouse neural crest cell (NCC) primary culture. Using the NCCmelb4 cell line, we investigated the occurrence of apoptosis in the cultured cells when KIT receptors were blocked by the monoclonal anti-KIT antibody (ACK2). Apoptosis following treatment with ACK2 was detected by DNA fragmentation assay, *in situ* apoptosis detection, and electron microscopy. We noted a decrease in extracellular signal-related kinase (ERK) and ribosomal S6 kinase (RSK) protein expression following ACK2 incubation. Western blot analysis and real-time quantitative RT-PCR revealed an apparent time-dependent reduction in Bcl-2 protein levels with respect to ACK2 within the NCCmelb4 cells. In terms of Bax expression, a difference was not found. Fas and caspase8 proteins increased time-dependently in proportion to ACK2 incubation. We noted apoptotic cell death upon addition of ACK2, with evidence of possible involvement of Bcl-2 and Fas in the induction of apoptosis. In contrast, no significant correlation between Fas ligand (Fas-L) expression and ACK2 was found. Fas activation appears to occur independent of Fas-L during ACK2-induced cell death. Therefore, we propose that Fas-L expression in NCCmelb4 cells does not play a major role in facilitating apoptosis. Furthermore, we hypothesize that these molecules combined with SCF/KIT play an important role in regulating the induction of vertebrate NCC apoptosis during embryogenesis.

Key words: Bcl-2/ERK/fas/fas ligand/neural crest cells/RSK
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Stem cell factor (SCF) induces its biologic effects via a tyrosine kinase receptor named KIT, which is expressed on melanocytes in the skin (Jiang *et al*, 2000). The proto-oncogene *c-kit* is a gene encoding a tyrosine kinase receptor and has been mapped to the *dominant white spotting* (*W*) locus. SCF is the ligand for KIT (*c-kit* protein) and has been mapped to the *Steel* (*Sl*) locus. According to previous phenotype analyses of *W* and *Sl* mice, it is clear that KIT and SCF play a crucial role in the development of hematopoietic cells, germ cells, and melanocytes. SCF increases melanocyte proliferation, differentiation, survival, chemotaxis, and secretion, as well as accumulation *in vivo* (Hermes *et al*, 2001). Activation of the KIT receptor in the presence of SCF indicates that SCF is essential to melanocyte proliferation and differentiation during the early embryonic stages (Hachiya *et al*, 2001). The major role of SCF in melanogenic phenomena is considered to be the targeting of KIT receptor-bearing cells, as evidenced by the migration of melanoblasts from the neural crest toward hair follicles via the epidermis.

Abbreviations: ERK, extracellular signal-related kinase; Fas-L, Fas ligand; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NCC, neural crest cells; RSK, ribosomal S6 kinase; SCF, stem cell factor; TNF, tumor necrosis factor

The activation of signal transduction pathways by growth factors, hormones, and neurotransmitters is mediated through two closely related MAP kinases, p44 and p42, designated extracellular-signal related kinase 1 (ERK1) and ERK2, respectively. During melanocyte development, SCF activates its receptor (KIT) and this triggers the activation of MAP kinase (Buscà and Ballotti, 2000). The 90 kDa family of ribosomal S6 kinases (RSK), designated RSK1, RSK2, and RSK3, are important signaling intermediates that mediate the response to a broad range of ligand-activated receptor tyrosine kinases. Upon activation, ERK phosphorylates the RSK, and they translocate to the nucleus where they phosphorylate and activate transcription factors involved in cell proliferation or differentiation, depending on the cell system. Apoptosis is a distinctive form of cell death that can result in the deletion of specific cell populations during physiologic processes such as embryonal development and clonal selection of lymphocytes. The Bcl-2 family of genes can either positively or negatively regulate programmed cell death. The Bcl-2 protein heterodimerizes with Bax (Bcl-2 associated X protein) in mitochondria, which is a potent mediator of programmed cell death. The Bcl-2/Bax ratio appears to determine whether some cells live or die (Korsmeyer, 1999). Several groups (Kim *et al*, 2000; van den Wijngaard *et al*, 2000) have demonstrated Bcl-2 expression in melanocytes,

whereas Bax was expressed in melanocytes but not in most melanoma lines (Bowen *et al*, 2003). Bcl-2-deficient mice turn gray with the second hair follicle cycle, implicating a defect in melanin biosynthesis. Recent studies have shown that activation of apoptosis machinery in germ cells involves the Fas/Fas-L (Fas ligand) system (Wang *et al*, 1998; Woolveridge *et al*, 1999). The Fas/Fas-L interaction has been implicated in the immune-privileged state of the eye, testis, and placenta. Fas (CD95 or APO-1) is a cell surface receptor that transduces apoptotic signals critical for immune homeostasis and tolerance (Lenardo *et al*, 1999; Wallach *et al*, 1999). It has a cytoplasmic death domain that binds with the Fas-associated death domain (FADD/MORT1) upon activation by Fas-L. FADD then induces caspase cascade activation, firstly caspase8 followed by caspase3. A protein termed caspase8 exhibits marked sequence homology with members of the caspase1/CED-3 family and binds to FADD. Caspase8 p20 reacts with the p20 subunit and precursor of caspase8.

Okura *et al* (1995) reported that a monoclonal anti-KIT antibody (ACK2) injection after birth causes normally black coat mice to change to various grades of white. Therefore, they suggested that melanocytes in newborn mice are KIT-dependent and undergo apoptosis when KIT receptors are blocked by ACK2 in the early days after birth. We previously demonstrated evidence that blocking the KIT/SCF interaction induces apoptosis in KIT-positive melanocyte precursors in a mouse neural crest cell (NCC) culture system (Ito *et al*, 1999). Furthermore, an immortal cell population of NCC from WB mice was established and designated as NCC-S4.1 cells (Kawa *et al*, 2000). From these NCC-S4.1 cells, we cloned a cell line named NCCmelb4 cells using a single-cell cloning method. They retain normal DNA ploidy and appear to be immature melanocytes, as they are positive for some marker molecules for melanocytes, but are negative against tyrosinase and dihydroxyphenylalanine (DOPA) (Kawakami *et al*, 2002; Watabe *et al*, 2002). Thus, this cell line appears to be ideal for studying factors that affect melanocyte development and melanogenesis. In the present study, we examine how ACK2 inhibits the proliferation of NCCmelb4 cells and induces apoptosis by DNA gel electrophoresis, immunohistochemical staining, and electron microscopy. Using the NCCmelb4 cell line, we investigate the effects of apoptosis-related molecules, Bcl-2, Bax, Fas, Fas-L, and caspase8 on apoptosis induced by ACK2 in immature melanocyte precursors.

Results

Inhibitory effects of ACK2 on the proliferation of NCCmelb4 cells We investigated the effect of ACK2 on the proliferation of NCCmelb4 cells (Fig 1). As controls, NCCmelb4 cells were incubated in a fetal calf serum (FCS) + minimum esential medium (MEM) + SCF-supplemented medium. When ACK2 was added, maximum growth was not observed. Growth was significantly retarded after 5 d in the presence of ACK2 ($p < 0.01$). ACK2 appeared to inhibit cell proliferation in a concentration-dependent manner (Fig 2).

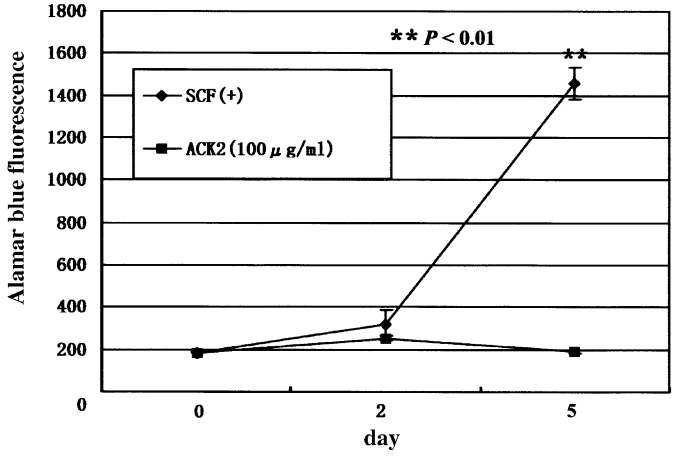

Figure 1
Inhibition of proliferation of neural crest cell (NCC)melb4 cells by anti-KIT antibody. NCCmelb4 cells were plated on 96-well plates at 2000 cells per well. The next day, anti-KIT antibody (ACK2) 100 μg per mL was added to some of the culture media (day 1). Cells were incubated with alamar Blue for 4 h and fluorescence was read on a Fluoroskan II microplate reader. Greatest growth of NCCmelb4 cells was seen in the presence of stem cell factor (SCF), with lower rates seen in the presence of ACK2.

Appearance of apoptotic cells We analyzed genomic DNA extracted from the NCCmelb4 cells incubated with or without 100 μg per mL ACK2 for 24, 48, or 72 h. DNA from the ACK2-treated cells exhibited a ladder-like pattern of oligonudeosome-sized DNA fragments, characteristic of apoptosis, in proportion to the incubation period (Fig 3). We also performed the Apop Tag *in situ* apoptosis detection assay (Fig 4*a*, *b*). Incubation with ACK2 for 48 h resulted in an increased number of apoptotic cells. As shown in Fig 4*c*, 48 h blocking the SCF/KIT interaction induced a large number of apoptotic cell to the maximum. Some cells had condensed nuclei with homogeneous and compacted chromatin, which are characteristics of apoptotic cells by electron microscopy (Fig 4*d*).

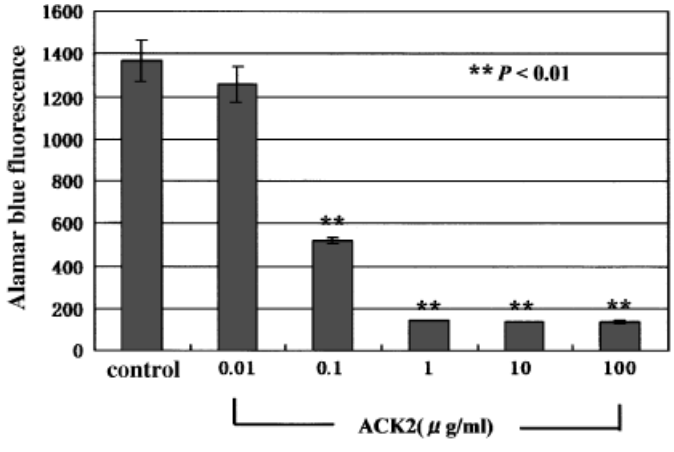

Figure 2
Effect of anti-KIT antibody on the proliferation of neural crest cell (NCC)melb4 cells. NCCmelb4 cells were plated at 2000 cells per well on 96-well plates, and incubated with various concentrations of anti-KIT antibody (ACK2). After 5 days, NCCmelb4 cell growth was significantly inhibited by concentrations of 0.01–100 μg per mL ACK2, in a concentration-dependent manner, compared with control wells ($^{**}p < 0.01$). Values represent means ± SD of eight determinations.

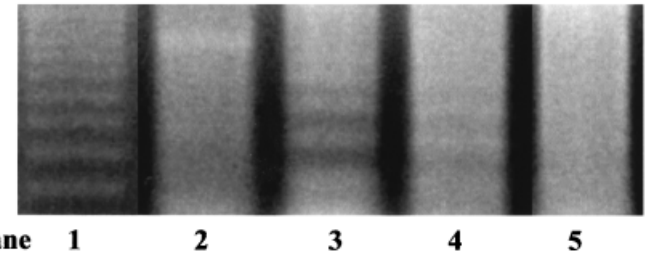

Figure 3
DNA fragmentation assay. Agarose gel electrophoresis was used to detect internucleosomal DNA cleavage in neural crest cell (NCC)melb4 cells with or without anti-KIT antibody (ACK2). Lane 1, ladder marker; lane 2, ACK2-treatment for 24 h; lane 3, treatment for 48 h; lane 4, treatment for 72 h; lane 5, no treatment. Apoptosis increased in a time-dependent manner.

Variations in apoptosis regulator proteins of NCCmelb4 cells After treatment with ACK2 for 24, 48, or 72 h, NCCmelb4 cell extracts were prepared and analyzed by western blotting. The activated ERK was detected using an antibody against the phosphorylated forms of ERK. The addition of extrinsic ACK2 gradually decreased the effect of the p-ESK protein and completely disappeared by 72 h (Fig 5*a*, *b*). Similarly, there was a significant decrease in phosphorylated RSK family proteins through ACK2-mediated apoptosis compared with normal conditions (Fig 5*c*, *d*). We then examined Bcl-2, a negative regulator of the mitochondrial apoptotic pathway, and found that Bcl-2 protein expression decreased time-dependently following ACK2 incubation, and completely disappeared by 72 h (Fig 6*a*). In contrast, no significant correlation between Bax expression and ACK2 was found (Fig 6*b*). Because Fas binding to its ligand, Fas-L, has been shown to trigger apoptosis in various cell types, we examined the expression in our cells. In a normal medium, NCCmelb4 cells did not express the Fas protein. Western blotting, however, revealed a gradual increase in Fas protein levels within the NCCmelb4 cells relative to the ACK2 treatment period (Fig 6*c*). On the other hand, Fas-L was strongly expressed in NCCmelb4 cells under normal medium conditions, whereas after ACK2 treatment, there was an apparent time-dependent reduction in Fas-L protein levels (Fig 6*d*). Caspase8 p20 subunit protein increased time-dependently in proportion to ACK2 incubation (Fig 6*e*).

Downregulation of Bcl-2 mRNA in ACK2-mediated apoptosis We examined the gene expression of Bcl-2 in NCCmelb4 cells using real-time quantitative RT-PCR. This approach offers the advantage of a broad dynamic range coupled with the ability to correct for quantity and quality of the sample. The RT-PCR experimental conditions were optimized to obtain an efficacy up to 90% of standard curves. Real-time RT-PCR revealed a time-dependent reduction in Bcl-2 mRNA levels within NCCmelb4 cells after ACK2 72 h treatment (Fig 7).

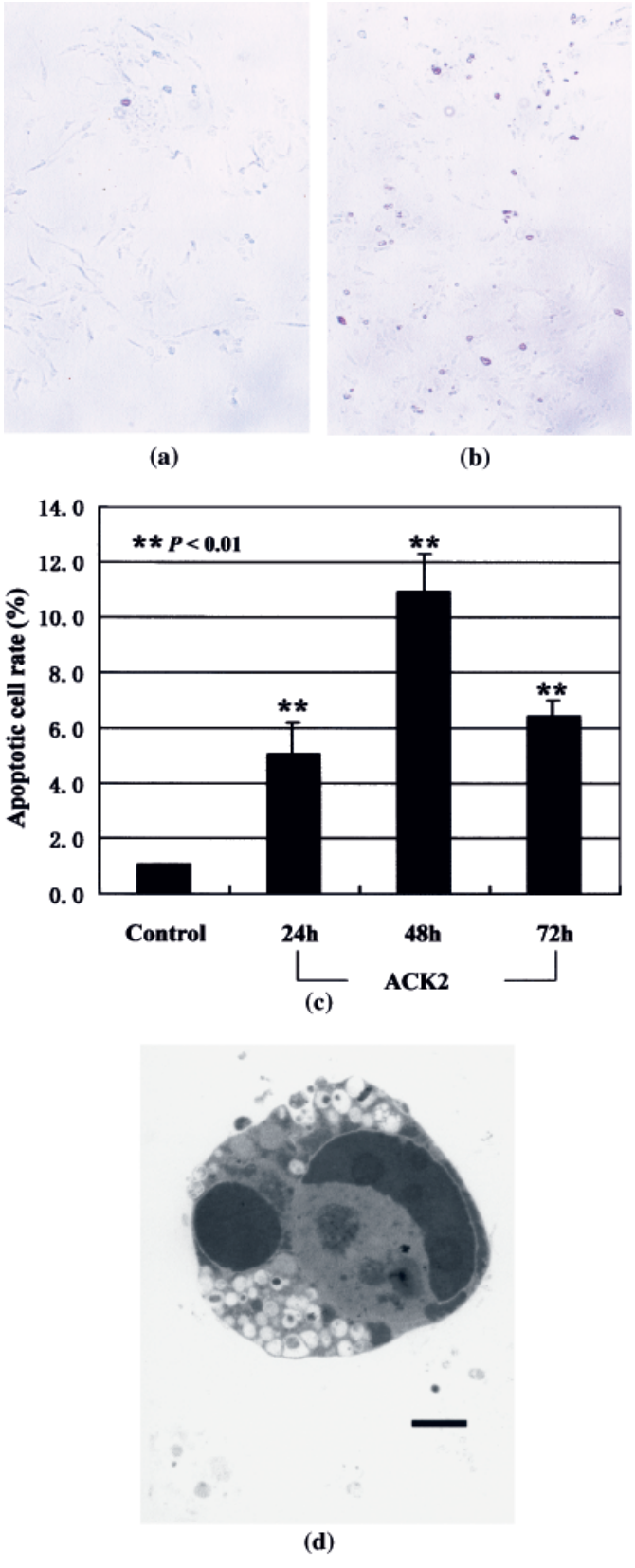

Figure 4
Increased apoptosis after anti-KIT antibody treatment. Neural crest cell (NCC)melb4 cells before (*a*) and after (*b*) 48 h of anti-KIT antibody (ACK2) incubation were stained using the Apop Tag *in situ* apoptosis detection kit. The apoptotic cell rate (*c*) was expressed as the percentage of apoptotic positive nuclei among 500 NCCmelb4 cells counted under light microscopy at high-power magnification. After treatment with ACK2 for 24, 48, or 72 h, the rate was significantly increased as compared to no treated control (**$p < 0.01$). Electron microscopic findings of apoptotic cells after 48 h anti-KIT antibody incubation (*d*). Apoptotic cells with scattered fragments of condensed nuclei contained melanosomes at immature stages. *Scale bar* = 1 μm.

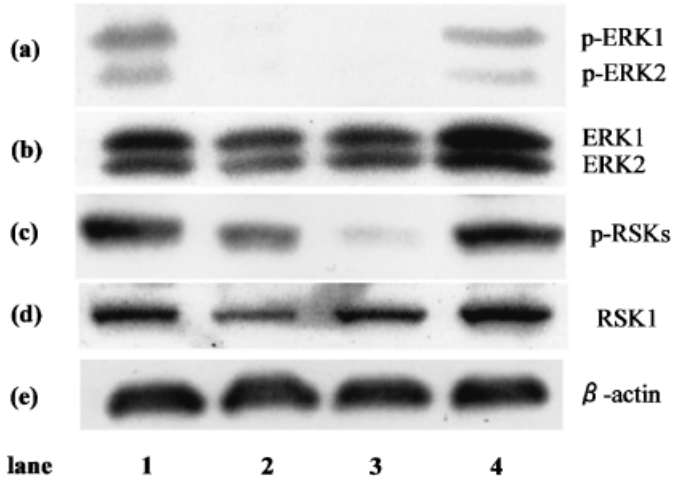

Figure 5
Western blot analysis of p-ERK (extracellular signal-related kinase) and p-RSK (ribosomal S6 kinase) family proteins in neural crest cell (NCC)melb4 cells incubated in anti-KIT antibody medium. ERK activation was detected using an antibody against phosphorylated ERK, p44 and p42, designed ERK1 and ERK2. NCCmelb4 cells were incubated for 24, 48, or 72 h in the presence or absence of anti-KIT antibody (ACK2) (100 μg per mL). p-ERK1 and p-ERK2 protein expression completely disappeared by 72 h (*a*, *b*). p-RSK1 (Thr 359/Ser 363)-R reacts with Thr-359 and Ser-363 phosphorylated RSK family proteins of mouse origin. Anti-KIT antibody (ACK2) induced a significant decrease in the p-RSK family proteins between ACK2-treated and control sample (*c*, *d*). (Lane 1, 24 h; lane 2, 48 h; lane 3, 72 h; lane 4, no ACK2 treatment.)

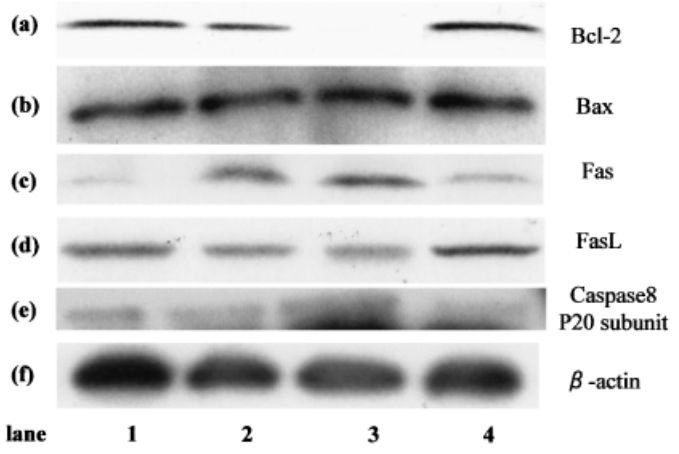

Figure 6
Western blot analysis of Bcl-2, Bax, Fas, Fas-ligand (Fas-L) and caspase8 p20 subunit in anti-KIT antibody (ACK2)-treated neural crest cell (NCC)melb4 cells. ACK2 (100 μg per mL) was added to the medium and incubation was performed for varying lengths of time (lane 1, 24 h; lane 2, 48 h; lane 3, 72 h). Cells were incubated in the absence of ACK2 as controls, lane 4. ACK2 decreased Bcl-2 expression in NCCmelb4 cells as revealed by western blotting. Bcl-2 (*a*) protein expression was lowest in lane 3. In terms of Bax (*b*) expression, no difference was found between ACK2 treated and control. Note the increased Fas (*c*) expression compared with the non-treated cells in a time-dependent manner. Fas-L (*d*) was weakly expressed at 48 and 72 h incubation as compared with control samples. Moreover, the addition of extrinsic ACK2 gradually increased the expression of caspase8 p20 subunit (*e*).

Discussion

Skin melanocytes are derived from NCC that migrate into the dermis and epidermis during embryogenesis. To explore the potential survival function of SCF during melanogenesis,

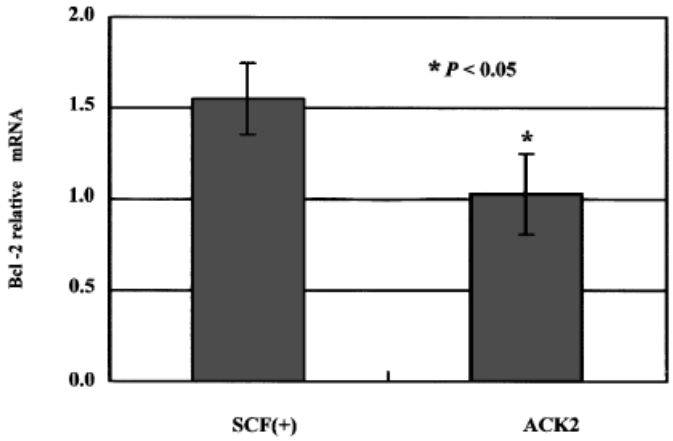

Figure 7
Real-time quantitative RT-PCR analysis of Bcl-2 transcript in neural crest cell (NCC)melb4 cells. ACK2 induced a significant time-dependent decrease in the mRNA transcripts of Bcl-2 according to RT-PCR ($^*p < 0.05$). Expression level of Bcl-2 was normalized to the amount of GAPDH mRNA.

NCCmelb4 cells were cultured, as an immature melanocyte precursor, in the presence of ACK2 (100 μg per mL) for 24, 48, or 72 h, and apoptosis was analyzed by DNA laddering. Apoptosis in ACK2 treatment was also confirmed by the TUNEL (Tdt-mediated d-UTP nick end labeling) method and electron microscopy. We found that ACK2 abolished the survival effect of SCF on NCCmelb4 cells in a time-dependent manner, and apoptosis in NCCmelb4 cell increased following treatment with ACK2. These findings suggest that the SCF/KIT system is involved in the regulation of germ cell apoptosis, such that more germ cells undergo apoptosis due to the blockade of SCF/KIT interaction in the presence of ACK2. In addition, western blotting revealed a reduction in p-ERK and p-RSK family proteins levels within the NCCmelb4 cells that seemed to be time-dependent with respect to ACK2. Of the various pathways that are activated by SCF/KIT, the pathway that comprises the series of sequentially activated ERK and phosphorylated RSK was found to promote cell survival and inhibit apoptosis in murine melanocyte precursors.

Bcl-2 family proteins are thought to act as upstream factor in the apoptotic machinery. Because Bcl-2 plays a crucial role in intracellular apoptotic signal transduction and displays both positive and negative regulatory effects on apoptosis, we investigated Bcl-2 in NCCmelb4 cells. Western blot and real-time RT-PCR analyses revealed a marked decrease in Bcl-2 expression as a result of ACK2 treatment. In contrast, Bax protein by ACK2-mediated apoptosis did not increase significantly compared with normal conditions. These findings indicate that apoptotic signaling inhibition by Bcl-2 is reduced in ACK2-treated NCCmelb4 cells. We previously showed that all-*trans* retinoic acid decreased Bcl-2 expression in NCCmelb4 cell and induced differentiation of murine melanocyte precursors (Watabe *et al*, 2002). These results suggest that the occurrence of apoptotic cell death in precursor cells undergoing early organogenesis may induce differentiation in the control of SCF-mediated mechanism. Increased anti-apoptotic Bcl-2 has been found in melanoma compared with melanocytes in some but not all studies (Selzer *et al*, 1998; Tang *et al*, 1998; Olie *et al*, 2002).

There is little information, however, on the expression of apoptotic regulators in immature melanocytes.

Fas and its ligand (Fas-L) are among the molecular mediators that trigger the apoptotic cascade. Fas is a surface (transmembrane) receptor that is a member of the tumor necrosis factor (TNF) receptor family. Fas mediates apoptosis in a wide variety of cell types. Fas-L is a type II transmembrane receptor that is also a member of the TNF family. As demonstrated *in situ*, the expression of Fas-L appears to be enhanced during progression of melanoma (Maeda *et al*, 1998). Fas-L is not found in normal melanocytes of the skin, indicating that Fas-L upregulation probably occurs during tumorigenesis (Redondo *et al*, 2002). Fas-L expression has been seen in various types of tumors, including melanoma, suggesting a novel immune escape mechanism (Hahne *et al*, 1996). The tumor counterattack hypothesis is discussed rather contradictorily in recent years. Eberle *et al* (2003) indicated that Fas-L overexpression triggered apoptosis in melanoma cells, whereas Fas overexpression could only sensitize cells for Fas-L-induced apoptosis. On the other hand, Raisova *et al* (2001) reported that mitochondria are the main orchestrators, whereas Bcl-2 and Bax play a central role in the regulation of Fas-induced apoptosis in human melanoma cells. Signalling by the TNF receptors had been thought to be mediated by the binding of the trimeric ligand TNF to three monomeric subunits of the receptor. But recent evidence suggests that an alternative model of TNF receptor signaling should be considered. TNF receptors, including Fas, appear to function as preformed complexes rather than as individual receptor subunits that oligomerize after ligand binding (Chan, 2000). This pre-ligand binding assembly domain (PLAD) is critical for assembly of functional receptor complexes on the cell surface. We detected Fas and Fas-L by western blot analysis in NCCmelb4 cells. This is a study of Fas and Fas-L expression in melanocyte precursors to the best of our knowledge. Surprisingly high levels of Fas-L expression were noted in murine immature melanocytes. Whereas Fas was upregulated in apoptosis associated with ACK2, there was a weak downregulation in Fas-L expression between the ACK2-treated and control sample. In addition, caspase8 p20 subunit expression was gradually increased in ACK2 incubation. These results suggest that Fas-L expression does not necessarily correlate with susceptibility to Fas-induced cell death in immature normal melanocytes. Therefore, it appears that an increase in Fas in NCCmelb4 cells, regardless of the presence of Fas-L, results in upregulated apoptosis. Furthermore, activation of both Bcl-2 and Fas may influence a cell's susceptibility to ACK2-mediated apoptosis. The interrelation between the SCF/KIT system and these apoptosis-related molecules in the regulation of germ cell apoptosis during melanocyte development remains an interesting topic for future studies.

Materials and Methods

Cell and culture conditions NCCmelb4 cells were established using a single-cell cloning method from an immortal cell population (NCC-S4.1) produced using NCC of WB mice (Kawa *et al*, 2000). The cells were grown in a humidified atmosphere of 5% CO_2 and 95% air at 37°C in Eagle's MEM (IBL, Maebashi, Japan) supplemented with 5% FCS (GIBCO, Grand Island, New York) and 50 ng per mL of recombinant mouse SCF (Kirin Brewery, Tokyo, Japan), unless indicated otherwise. Under these culture conditions, the NCCmelb4 cells were KIT-positive, tyrosinase-negative melanocyte precursors that remained at an immature undifferentiated stage. Electron microscopic observation revealed that these cells contained only stage I melanosomes with no advanced-stage melanosomes.

Proliferation assay NCCmelb4 cells were plated at 2000 cells per well on 96-well plates. The next day, various concentrations of anti-KIT monoclonal antibodies (ACK2; 0–100 μg per mL) were added to the culture medium including SCF (day 0). On days 0, 2, and 5, the cells were incubated with alamar Blue (Trek Diagnostic Systems, Westlake, Ohio) for 4 h at 37°C and fluorescence was determined using a Fluoroskan II microplate reader (Labsystems, Helsinki, Finland).

DNA analysis by agarose gel electrophoresis and immunohistochemistry NCCmelb4 cells were incubated in the presence of 100 μg per mL ACK2 for 24, 48, and 72 h or in the absence of ACK2. Agarose gel electrophoresis (Apoptosis Ladder Detection kit, Wako, Osaka, Japan) was used to detect internucleosomal DNA cleavage in whole ACK2 cells added to the medium. For the detection of apoptotic cells, cell cultures were stained with the Apop Tag *in situ* apoptosis detection kit (Oncor, Gaithersburg, Maryland). The Apop Tag kit is a modification of the TUNEL method that can detect DNA fragments caused by apoptosis.

Electron microscopy NCCmelb4 cells were incubated in ACK2-treated for 48 h. Control cells were not treated. Cell colonies were fixed overnight on a slide culture plate with 2% glutaraldehyde at 4°C. After being washed in a buffer solution, the cells were post-fixed with 1% OsO_2 for 1 h on ice, washed in distilled water, dehydrated with graded ethanol solutions, and then finally embedded in epoxy resin. Ultrathin sections were double-stained with uranyl acetate and lead citrate, and observed under an electron microscope (JEM-1200EX, JEOL, Tokyo, Japan).

Western blotting NCCmelb4 cells were cultured with MEM + SCF + FCS. ACK2 was then added to the culture medium, to a final concentration of 100 μg per mL, and cells were cultured for 24, 48, or 72 h. Cells were detached with Trypsin/EDTA and collected by centrifugation. The cell pellets were treated with an extraction buffer containing 1 M Tris-HCl pH 7.2, Nonidet P-40, 10% SDS and Complete Mini (Roche Diagnostics, Mannheim, Germany). The cell extract was obtained at 4°C for 2 h. Samples were placed on ice and analyzed for protein concentration using the DC Protein Assay (Bio-Rad Laboratories, Tokyo, Japan). Aliquots of the sample protein, 20 μg each, were run on a Tris-Glycine gel (Bio-Rad Ready Gels J, Bio-Rad Laboratories). After electrophoresis, proteins were transferred onto a polyvinylidene difluoride membrane (Immobilon-p, Millipore, Bedford, Massachusetts) and incubated with ERK, p-ERK, RSK1, p-RSK1 (Thr 359/Ser 363)-R, Bcl-2, Bax, Fas, Fas-L, and caspase8 p20 subunit (1 μg per mL, Santa Cruz Biotechnology, Santa Cruz, California, each) overnight at room temperature (RT). Horseradish peroxidase donkey anti-rabbit IgG (Amersham Biosciences, Piscataway, New Jersey) was used as a secondary antibody. Antibody to mouse β-actin was from Abcam Ltd., Cambridgeshire UK. Visualization of the immunocomplexes was performed by enhanced chemiluminescence using the ECL kit (Amersham Pharmacia Biotech, Dübendorf, Switzerland), followed by exposure to Hyperfilm ECL (Amersham Biosciences).

RNA extraction and real-time quantitative RT-PCR analysis Total RNA was extracted from the cells using an RNA isolation kit (Promega, Madison, Wisconsin) as described by the manufacturer. Total RNA extraction was performed with RNase-free RQ1 DNase (Promega) at 37°C for 30 min. RNA samples (1 μg) were subjected to reverse transcription using MMLV Reverse Transcriptase (Invitrogen, Leek, the Netherlands). For Bcl-2 amplification, primers

were 5′-CCTGTGGATGACTGAGTACC-3′ (sense) and 5′-GAGACAGCCAGGAGAAAT-3′ (antisense). To amplify and quantify target cDNA, 2 μL aliquots of reverse transcribed samples were subjected to PCR amplification on the LightCycler (Roche Diagnostics) in 20 μL containing a final concentration of 0.5 μmol per L of each specific primer, 3 mmol per L of $MgCl_2$, and 2 μL of ready-to-use LightCycler DNA Master SYBR Green I. The reaction conditions consisted of initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 10 s, and extension at 72°C for 6 s. PCR product specificity was verified using a melting curve procedure and by electrophoresis through a 2% agarose gel. Detection and quantification of the expression of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were amplified using specific oligonucleotides (5′-TGC(A,C)TCCTGCACCACCAACT-3′, 5′-(T,C)GCCTGCTTCACCACCTT-3′) and carried out as a control to confirm RT-PCR quality. Quantitative analysis was performed using LightCycler software ver. 3.5 (Roche Diagnostics), with real-time fluorogenic detection. Expression of target genes was measured in triplicate and then normalized to GAPDH expression level.

Statistical analysis Significant differences were evaluated by a two-tailed, unpaired *t* test. The level of significance was set at $p < 0.05$ in all cases. In addition, statistical analysis of the apoptotic cell rate was determined using the Mann–Whitney *U*-test. All experiments were repeated more than three times.

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