Regulation and dysregulation of hematopoiesis by a cytokine-induced antiapoptotic molecule anamorsin

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Many growth factors and cytokines have a crucial role in hematopoiesis, and the cytokine-receptor system is known to involve in normal and abnormal hematopoiesis. Upon binding to receptors, cytokines initially induce activation of cell surface tyrosine kinases that transmit mitogenic and anti-apoptotic signals through simultaneous activation of downstream signaling molecules including Ras/MAPK, PI3-K/Akt, and STATs. However, it is not fully understood yet how cytokines mediate anti-apoptotic effects. To define molecule(s) that mediates anti-apoptotic effect of cytokine and confer resistance to apoptosis induced by cytokine deprivation, we have established a subline named Ba/F3-Ad that can grow and survive under IL-3-deprived conditions from a murine IL-3-dependent cell line, Ba/F3. In order to identify the molecule(s) that conferred the resistance to factor-deprived apoptosis on Ba/F3, we performed the expression cloning by constructing a retroviral cDNA library from IL-3-starved Ba/F3-Ad. In short, we infected the retrovirus library into parental Ba/F3 (5×105 clones, the average size of the inserts, 1.3 kb; the infection efficacy was estimated at about 35%), and screened the clones that can survive under IL-3-deprived condition. After isolation of several clones that can survive under IL-3-deprived condition, we isolated the integrated cDNA from genomic DNA extracted from one clone by the PCR method.

By sequencing the integrated cDNA, we found that the coding region of murine AM cDNA consists of 930 bp. Comparison with the DNA data base search revealed that the sequence of AM does not exhibit homology with any known anti-apoptotic molecules, including Bcl-2 family proteins, caspase inhibitors, or signal transduction molecules. Also, we found a human homologue of AM in EMBL/GenBank data libraries, which revealed 82.6% similarity to murine AM at a DNA level and 81.9% similarity at an amino acid level. Human homologue of AM was originally found by Lotfus et al. as a molecule with unknown function on chromosome 16. AM encodes an about 37-kDa protein and the protein sequence data base indicates that AM had generic methyltransferase motif around amino acids 60–99.

At first, we examined whether AM was indeed involved in the resistance to factor-deprived apoptosis of Ba/F3-Ad. For this purpose, we stably expressed murine AM cDNA in parental Ba/F3 cells, and investigated their sensitivities to IL-3-deprived apoptosis. In control Ba/F3 cells, the subdiploid fraction formed from apoptotic cells emerged as early as 24 h after IL-3 depletion, which was effectively reduced in AM-transfected Ba/F3 cells (% of apoptotic fraction: control Ba/F3, 25% at 24 h, 57% at 36 h, 87% at 48 h; AM-transfected Ba/F3, 3% at 24 h, 4% at 36 h, 12% at 48 h). In agreement with this finding, the activation of caspase-3, which is detected as a conversion of left-sided peak to right-sided peak in fluorescent intensity, was significantly suppressed in AM-transfected Ba/F3 cells as compared to that in control Ba/F3 cells. However, it should be noted that AM alone could not support the growth of Ba/F3 cells, since proliferating cells drastically decreased in AM-transfected Ba/F3 cells under IL-3-deprived condition (% of the cells in S-G2/M phase before and after IL-3 depletion: 68% at 0 h vs. 10% at 48 h). We also confirmed that AM can confer resistance to apoptosis caused by IL-3 depletion in another murine IL-3-dependent cell line, 32D.
Next, we examined the expression profile of human AM homologue in various organs using MTA panels. AM was expressed ubiquitously in various tissues, especially with high expression levels in heart, liver and pancreas. As for hematopoietic tissues, it was abundantly expressed in fetal liver and spleen. Also, we found that the expression of AM was detectable from the early stages (7 day) of embryogenesis by the PCR method.

Since AM was supposed to exert anti-apoptotic effects in Ba/F3-Ad under IL-3-deprived condition, we examined its expression levels in parental Ba/F3 and Ba/F3-Ad after IL-3 depletion. As expected, AM was still expressed in Ba/F3-Ad after 18-h IL-3 deprivation, while its expression was hardly detectable in parental Ba/F3 cells. However, the addition of IL-3 recovered it expression in parental Ba/F3 cells from after 3 h. We next investigated the expression of AM was also regulated by other cytokines in Ba/F3 cells. After IL-3 depletion, clones of Ba/F3 each expressing the receptors for EPO, SCF (c-Kit), and TPO (c-Mpl) were cultured with the corresponding cytokines for the time indicated. EPO, SCF, and TPO individually induced the expression of AM as efficiently as IL-3, suggesting that the expression of AM would be regulated by common signaling molecule(s) shared by various cytokines. Based on the fact that Ras is activated by various cytokines and was constitutively activated in Ba/F3-Ad, we speculated that Ras might control the expression of AM in Ba/F3 cells. To examine this possibility, we stably expressed oncogenic H-ras (H-rasG12V) in Ba/F3 cells. Also, we prepared the clone from Ba/F3, in which dominant negative H-ras (H-rasS17N) was inducibly expressed by the IPTG treatment. In Ba/F3 cells transfected with H-rasG12V, the expression of AM was maintained after IL-3 depletion for up to 36 h, while its expression declined to an undetectable level in the mock clone transfected with an empty vector. Furthermore, the induced expression of H-rasS17N led to the reduction of AM expression even in the presence of IL-3. Collectively, these results suggest that the expression of AM is completely dependent on cytokine stimulation, and, at least partially, regulated by Ras signaling in Ba/F3 cells. Next, we investigated the intracellular localization of AM by an immunofluorescence staining with an anti-AM monoclonal antibody (mAb). AM was exclusively localized at cytoplasm in Ba/F3 cells irrespective of the stimulation with IL-3.

To assess in vivo roles of AM, we tried to generate AM null (AM-/-) mice. We constructed a targeting vector, in which the first exon was replaced by the neomycin-resistant cassette (a positive selection marker). The vector also included the diphtheria toxin as a negative selection marker. The targeting vector was introduced into an ES cell line R1 by electroporation, and the transfected cells were cultured with 150 μg/ml of G418. We confirmed the homologous recombination in the selected ES cell lines with Southern blot and PCR analyses. The wild type allele was detected as a 5-kb SacI fragment in Southern blot analysis with a 5’ flanking probe, while the targeted allele was detected as a 13-kb SacI fragment. Also, in PCR analyses, the 310-bp fragment was amplified from the wild type allelic genomic DNA with a primer pair D/D, while the 730-bp fragment was amplified from the targeted allelic genomic DNA with a primer pair D/D. To generate chimeras, we injected three ES cell lines, in which the homologous recombination was confirmed, into blastocysts of C57BL/6J mice. Then, male mice with a high degree of chimerism were crossed with C57BL/6J females to generate AM+/− mice. Genotyping was performed by Southern blot and PCR analyses using tail- and embryo-derived DNA. Finally, we confirmed that the expression of AM protein in the limb was partially reduced in AM+/− embryos and completely lost in AM−/− embryos by Western blot analysis using an anti-AM mAb.

Genotypic analysis of embryos from AM+/− mice intercrosses revealed that AM−/− embryos started to die between E12.5 and E14.5; the rate of dead AM−/− embryos increased after E14.5 (0% until E12.5, 18.8% at E14.5, 36.4% at E16.5, and 44.4% at E18.5); and all AM−/− mice died at birth, suggesting that AM−/− mice expire in late gestation. AM−/− embryos were apparently smaller in body size than AM+/+ embryos, while AM+/− embryos displayed phenotypes similar to AM+/+ embryos. Despite no significant difference in the formation of blood islands in yolk sac, the fetal liver (FL) and spleen of AM−/− embryos were remarkably smaller than those of AM+/+ embryos; the size of FL of AM−/− embryos was almost one third of that of AM+/+ embryos, and the spleen of AM−/− embryos appeared scar. Furthermore, AM−/− embryos later than E14.5 looked anemic; AM−/− embryos at E18.5 showed almost half values of red blood cells (RBC), hemoglobin (Hb) and hematocrit (Ht) in the peripheral blood (RBC: 365±50.8, 335.5±50.6 and 210±67.0×10⁴/mm3; Hb: 11.7±1.5, 10.6±1.3 and 6.7±2.3 g/dl; Ht: 39.7±5.8, 37.8±5.3 and 26.3±7.6% in AM+/+ (n=10), AM+/− (n=22) and AM−/− (n=8) embryos, respectively). Moreover, RBC of AM−/− embryos were macrocytic (mean corpuscular volume in AM+/+, AM+/− and AM−/− embryos were 108.8±4.3, 113.1±9.5 and 128.2±16.6, respectively). In addition to the defect in hematopoietic organs, heart walls of AM−/− embryos were thinner than those in AM+/+ or AM+/− embryos, whereas AM−/− embryos displayed no apparent macroscopic or histological abnormalities in other organs.

In order to clarify the mechanism of anemia seen in AM−/− embryos, we analyzed the FL, the main
hematopoietic organ at late embryonic days. The number of morphologically identifiable small-sized erythroid cells (erythroblasts) with condensed chromatin nucleus was apparently reduced in FL of AM−/− embryos compared to that of controls. Furthermore, erythroblasts were larger in AM−/− embryos than in AM+/+ embryos, and more mature erythroblasts (i.e., polychromatic and orthochromatic erythroblasts) markedly decreased in AM−/− embryos. In addition, TUNEL assays showed that a substantial fraction of FL cells led to apoptosis at E14.5 in AM−/− embryos, while it was hardly detected in AM+/+ embryos. To determine which type of cells undergo apoptosis, we performed flow cytometric analysis using AM+/+ and AM−/− FL cells at E14.5. Although Annexin V-positive apoptotic cells were scarcely (only 0.5%) detected in AM+/+ FL cells, 60.2% of the isolated cells were positive for Annexin V in AM−/− FL. Furthermore, most importantly, almost all of these apoptotic cells were Ter-119-positive erythroid cells but not Ter-119-negative cells mainly composed from hepatocytes. Since neither the absolute number of hematopoietic stem/progenitor cells (CD34+CD44− or CD34 low CD44 high) nor that of very immature proerythroblasts (Ter-119+c-kit+) did not decrease in AM−/− FL, it was speculated that immature hematopoietic cells of AM−/− mice may succumb to apoptosis, and fail to attain terminal differentiation.

We next examined whether hematopoietic stem/progenitor cells of AM−/− FL cells could survive, proliferate and differentiate in response to cytokines in vitro. When E14.5 AM+/+, AM+/− or AM−/− FL cells were cultured in methylcellulose with the combination of cytokines SCF, IL-3, IL-6 and EPO, the number of myeloid (granulocyte/macrophage and granulocyte) colonies formed from AM−/− FL cells was one third to one fourth of those by AM+/+ FL cells. Notably, AM−/− FL cells gave rise to little or no mix (myeloid/erythroid) and erythroid colonies, while these colonies did develop from AM+/− and AM+/+ FL cells. Furthermore, BFU-E colony did not develop when E14.5 AM−/− FL cells were cultured with SCF and EPO (Fig. 5E). These results suggest that AM is indispensable for cytokine-dependent survival and growth of hematopoietic stem/progenitor cells in vitro, especially with erythroid lineage.

To characterize the anti-apoptotic function of AM, we compared the gene expression profiles between AM−/− and AM+/+ FL cells at E14.5 using a cDNA microarray. Among 4289 genes, including Bcl-2 family, caspases, cytokines and signal transduction molecules, 184 genes were significantly down-regulated and 40 were up-regulated in AM−/− FL. Concerning apoptosis related genes, Bcl-xL and Jak2 were down-regulated most significantly. We also confirmed that their expression was decreased in AM−/− FL cells compared with AM+/+ FL cells by semiquantitative RT-PCR assays. Furthermore, AM was found to express in a part of leukemia and malignant lymphoma cells at a significantly high level.

These results suggest that AM may play a crucial role in hematopoiesis through mediating anti-apoptotic effects of various cytokines, and also that AM may contribute to abnormal growth of leukemia/lymphoma cells.