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Overexpression of Survivin Initiates Hematologic Malignancies In Vivo

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Abstract

Survivin is an IAP family member that plays an essential role in cellular proliferation as an essential component of the chromosome passenger complex. Survivin is highly expressed in embryos and in proliferating adult tissues, but it is not expressed in most differentiated cells. During tumorigenesis, however, survivin expression is dramatically upregulated. Although many studies have shown that survivin is required for cancer cells, the extent to which survivin contributes to the initiation of tumors is unknown. Here we show that transgenic mice that overexpress survivin in hematopoietic cells are at an increased risk of hematologic tumors. In examining how survivin might contribute to tumorigenesis, we observed that hematopoietic cells engineered to overexpress survivin are less susceptible to apoptosis. We conclude that survivin may promote tumorigenesis by imparting a survival advantage to cells that acquire additional genetic lesions.

Introduction

Survivin, the smallest member of the inhibitor of apoptosis protein (IAP) family, has long been recognized as a prognostic and diagnostic marker of various cancers, as it is expressed at very low levels in most adult tissues, but elevated in tumors. High levels of survivin have been observed in many types of solid tumors, such as lung, ovarian, breast, gastric, prostate, and pancreatic cancers (1). Overexpression of survivin is shared by hematological malignancies, including acute leukemias, anaplastic and diffuse large cell lymphoma, myelodysplastic syndromes (MDS), myeloma and a variety of pediatric blood cancers (2-7).

Survivin is an IAP family member, but whether it acts to inhibit apoptosis is controversial. Although several studies have suggested that survivin inhibits apoptosis (e.g. (8)), other reports have shown that cells lacking survivin die from mitotic catastrophe, the inability to complete mitosis (9). Indeed, an essential role for survivin in cytokinesis has been well defined. Survivin associates with Aurora B kinase, INCENP, and Borealin to form the

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chromosome passenger complex (CPC), which actively participates in mitosis (10, 11). Although survivin is expressed in a cell cycle dependent manner in normal proliferating cells, it is expressed in a cell cycle independent manner in cancer cells, suggesting that its high expression in tumors is not merely a consequence of the proliferative index (12).

Survivin is required for primitive hematopoiesis, proliferation and/or survival of hematopoietic stem and progenitor cells, erythroid cells, T-cells and activated neutrophils (13-18). Moreover, survivin is necessary for proliferation and acute leukemia induced by expression of the internal tandem duplication of FLT3 (19). The role of survivin in the early stages of hematopoietic neoplasms, however, has not been addressed. To determine whether overexpression of survivin is an initiating event in leukemia, we treated transgenic mice that overexpress survivin (GATA1-Sur) with the DNA alkylating agent, N-ethyl-nitrosourea (ENU). Here, we report that GATA1-Sur mice develop hematologic malignancies at an increased rate and with shorter latency than wild-type littermates. Furthermore, we show that splenocytes from transgenic mice as well as primary T-cells engineered to overexpress survivin exhibit modest reductions in spontaneous and induced apoptosis. Our results suggest that increased expression of survivin contributes to transformation of hematopoietic cells by promoting a subtle survival advantage.

Methods

Mice

GATA1-Sur mice, which express survivin under the control of the GATA-1 promoter, have been reported (20). Cohorts of transgenic and non-transgenic CD1 littermates were treated with a single intraperitoneal injection of ENU (100 mg/kg) at one month of age. Peripheral blood counts were determined using a HemVet950 (Drew Scientific, Oxford, CT). Balb/C mice were purchased from Jackson laboratories. Animal studies were approved by the Northwestern University IACUC.

Flow Cytometry

Single cell suspensions of bone marrow and spleen cells were incubated with various lineage specific antibodies and processed as described (21). Flow data were acquired with an LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo software (TreeStar). Cell cycle and apoptosis of primary splenocytes from wild-type and transgenic animals were measured as described (22).

Primary murine T cell experiments—T cells were enriched from spleens of BALB/c mice using EasySep negative selection mouse T cell enrichment kit (Stem Cell Technologies) and plated onto anti-mouse CD3e antibody (eBioscience clone 145-2C11) coated 12 well plates along with 5 µg/ml anti-mouse CD28 antibody (eBioscience clone 37.51) for stimulation. After 24 hours, cells were transduced with MIGR1 (encoding GFP alone) or MIGR1-Survivin (encoding Survivin and GFP) retroviruses and were incubated at 37°C under stimulation conditions. For proliferation assays, T cells were labeled with cell trace violet (Invitrogen) 24 hours after viral transduction and the GFP positive cells were analyzed by flow cytometry for proliferation 24, 48 and 72 hours later. To test for

susceptibility of these cells to apoptosis 24 hours after transduction, the cells were treated with drugs for 6-8 hours and stained with annexin V-cy5 (Biovision) and SytoX Blue (Invitrogen) and analyzed by flow cytometry. Cell lysates were prepared at 48 hours after transduction for western blot analysis.

Histology

Tissues were fixed and processed by standard protocols. Slides were photographed at 200x or 400x original magnification on a DM 4000B microscope with a DFC320 camera and captured with DFC Twain software version 6.6.0 (Leica Microsystems, Ltd, Switzerland).

Statistical Analysis

Evaluation of differences in lifespan was performed with the Log rank (Mantel-Cox) test. The Student's t-test (two-sided, equal variance) was used to compare apoptosis in control and survivin overexpressing primary T cells and in control versus transgenic splenocytes.

Results

We previously generated transgenic mice that express full-length human survivin cDNA under the control of the GATA-1 promoter (GATA1-Sur mice) and demonstrated that survivin was upregulated in both the erythroid and megakaryocyte lineages (20). Multiple reports have shown that expression driven by this GATA-1 promoter construct (Fig. 1A) is not restricted to erythroid cells and megakaryocytes, but is active more widely in hematopoietic cells (23-25). Indeed, quantitative RT-PCR analysis revealed that survivin mRNA was elevated 2-3 fold in B and T cells of transgenic mice as compared to controls (data not shown). Moreover, western blot analysis revealed that survivin expression was markedly upregulated in bone marrow and spleen cells of transgenic mice (Fig. 1B).

Overexpression of survivin in GATA1-Sur mice did not alter erythroid/megakaryocyte development or lead to spontaneous tumor formation (20). To investigate whether overexpression of survivin contributes to initiation of tumorigenesis and cooperates with secondary mutations to cause disease, we treated GATA1-Sur transgenic mice and non-transgenic littermates with ENU. Mice were monitored on a regular basis by observation and measurement of body weight and routine analysis of complete blood counts. Animals were sacrificed when body weight loss reached 20% or when peripheral blood counts indicated the presence of a hematological malignancy. Analysis of the survival data revealed that there was a significantly shorter latency for development of hematologic malignancies in the transgenic mice as compared to the wild-type littermates (Fig 1C). Within one year, 8 of 21 transgenic, but only 1 of 20 wild-type littermates developed disease and were euthanized. Moreover, the spectrum of disease that developed in the transgenic mice differed from that observed in non-transgenic mice. Whereas only one of the wild-type littermates developed a solid tumor at forty-seven weeks of age, all of the survivin transgenic mice that became sick presented with lymphoma. Three of the transgenic mice developed high-grade lymphoma with prominent tumor infiltration of the bone marrow, spleen, liver and thymus. The disease in these animals (Group A, exemplified by mouse #356) was accompanied by marked splenomegaly, elevated white blood cell count, anemia and thrombocytopenia (Fig 1D). In

contrast, five transgenic animals developed lymphoma that was not accompanied by splenomegaly or significant changes in peripheral blood counts (Group B, exemplified by mouse #315).

Analysis of stained histological sections revealed the presence of high-grade aggressive tumor in the thymus with involvement of the bone marrows and infiltration into the spleens and liver of mice from Group A (e.g. #356, Fig 2). These tumors were comprised of intermediate to large cells with a fine vesicular chromatin, high mitotic activity and conspicuous cellular debris – all indicative of a high cellular turnover. The second group of diseased animals displayed thymic involvement by tumor that was cytologically identical to the mice in Group A. However, the bone marrow and spleen in these mice demonstrated excessive hematopoiesis, including an abundance of erythroid and megakaryocytic precursors in the bone marrow and spleen, but no tumor cells in these organs (e.g. #315, Fig 2). Moreover, there was no infiltration of the liver. Western blotting confirmed that survivin was highly expressed in the spleens of diseased mice (Fig 3).

Next, we performed immunophenotyping of cells collected from the spleen, bone marrow and thymus of diseased transgenic and control mice. In both groups of animals the tumor cells did not express any of the myeloid antigens evaluated (data not shown). Flow cytometry confirmed the presence of an aberrant population of T cells in the bone marrow and spleen of animal 356 (Fig 4A). These cells consistently expressed CD8, with a subset co-expressing CD4. In contrast, consistent with the histology, we failed to detect appreciable numbers of T cells within the bone marrows and spleens of Group B mice. However, analysis of T cells within the thymus of mouse 315 revealed a prominent shift in the immunophenotype of this lineage: the majority of cells in the diseased mice stained for CD4, but not CD8 (Fig 4B). Taken together, these results indicate that all of the diseased survivin transgenic mice developed morphologically aggressive tumors that were derived from T-cells. The difference in the peripheral phenotype of the tumors in the two groups of animals may reflect the different stages of the same malignant process.

To investigate whether the secondary events caused by ENU occurred in known lymphoma or leukemia oncogene or tumor suppressors, we sequenced *Notch1* (HD and PEST domains), *Fbw7*, *Jak2*, and *Ikzf1* in spleen samples from diseased animals. We failed to find mutations in these genes in any of the murine tumor samples (data not shown).

As a way to determine how overexpression of survivin contributes to tumor initiation, we assayed the effect of ectopic survivin expression on apoptosis and cell cycle of two relevant cell populations. First, splenocytes collected from GATA1-Sur transgenic mice and wild-type control littermates were cultured ex vivo in the presence of etoposide or bcl-2 inhibitor. Transgenic cells showed a small, but significant, reduction in spontaneous death and apoptosis in response to etoposide (Fig 5A, B). Transgenic cells also showed a slight change in cell cycle parameters: GATA1-Sur splenocytes displayed a small but significant decrease in the proportion of G1 cells when cultured ex vivo (Fig 5C, D).

Second, splenic T cells were purified from wild-type Balb/C mice, electroporated with a survivin-IRES GFP expression construct or with GFP alone and used for assays of apoptosis

and proliferation. Western blot analysis revealed that survivin was overexpressed between 2 and 4-fold (Fig 6A). To measure the effect of survivin on apoptosis, cells were treated with various inducers of cell death 24 hours after transduction. Survivin expressing T-cells displayed significantly reduced death in response to a JAK2 inhibitor and a trend towards reduced apoptosis when cultured with an AKT inhibitor or trichostatin A (Fig 6B,C). Separately, aliquots of cells were cultured with cell trace violet 24 hours after transduction, and proliferation was evaluated at 24, 48 and 72 hours by flow cytometry. We failed to detect any appreciable differences in proliferation of these cells as measured by dye dilution (Fig 6C). Thus, in accord with multiple published studies that describe an anti-apoptotic role for survivin in tumors (8, 26), we conclude that survivin may contribute to tumorigenesis by conferring a subtle survival advantage to cells.

Discussion

Although not expressed in most adult tissues, survivin is highly expressed in proliferating cells, such as hematopoietic progenitor cells and erythroid cells. High expression of survivin is also seen in nearly all tumors and is frequently associated with poor prognosis (27). Previous studies to characterize the role of survivin in cancer have primarily focused on its anti-apoptotic activity and on its requirements for continued growth of tumors. Multiple groups have shown that reduction in survivin expression, by anti-sense, knockdown, or pharmacologic inhibition of gene expression, disrupts tumor growth. Indeed, survivin has been proposed to be a global target of tumor suppression networks (28). Several anti-survivin therapies, including the peptomimetic sheperdin, transcriptional repressors, antisense and anti-survivin immunotherapy are under investigation as novel anti-cancer therapies (8).

Our results suggest that overexpression of survivin may precede the development of hematologic malignancies and facilitate tumor formation. Of note, previous reports have suggested that overexpression of survivin facilitates the development of skin cancer. In one study, keratinocytes that overexpress survivin were shown to exhibit resistance to UVB-induced apoptosis in vitro and in vivo (29). In another study, keratin 14-survivin transgenic mice were found to show diminished regression of papilloma and increased conversion of papilloma to skin cancer (30). Our study is the first, however, to demonstrate a direct, causal relationship between increased survivin expression and development of hematologic malignancies.

Based on the increased susceptibility of GATA1-Sur mice to ENU-induced tumorigenesis, we conclude that survivin contributes to the initiation of neoplastic events. Although altered expression of mitotic regulators, such as survivin's partner aurora-B, increases the risk for carcinogenesis by induction of aneuploidy or tetraploidy (31, 32), our findings suggest that overexpression of survivin may contribute to cancer by promoting increased protection from cell death. The effect on survival conferred by ectopic survivin was modest, consistent with the lack of expansion of any particular hematopoietic lineage in healthy transgenic mice. Such a modest survival advantage, however, could certainly confer increased susceptibility to oncogenesis induced by subsequent genetic mutations. Finally, even though we failed to detect cytogenetic abnormalities in the two T-cell tumors analyzed (data not shown), we

cannot rule out the possibility that increased levels of survivin may induce aneuploidy. Based on our model that increased expression of survivin can facilitate tumor development, quantification of survivin expression in peripheral blood cells may be a useful predictor of future hematological malignancies.

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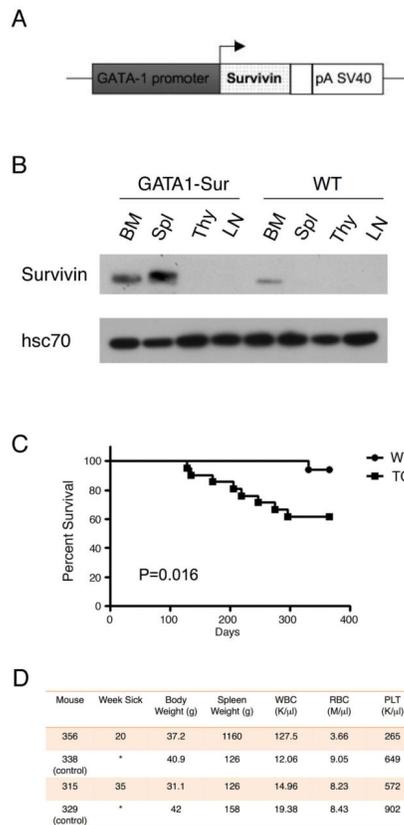


Figure 1. ENU-treated GATA1-Survivin transgenic mice are at increased risk of hematologic malignancies

A) Schematic of the GATA1-Sur transgene construct. B) Western blot of survivin expression in bone marrow (BM), spleen (Spl), thymus (Thy) and lymph node (LN) of GATA1-Sur transgenic mice and wild-type littermates. Hsc70 was used as a loading control. C) Survival analysis revealed that ENU-treated GATA1-Sur transgenic mice (TG) had a significantly accelerated disease progression as compared to ENU-treated wild-type littermates (WT). D) Hematopoietic indices and body and spleen weights of two representative transgenic mice (356 and 315) and their respective controls (338 and 329).

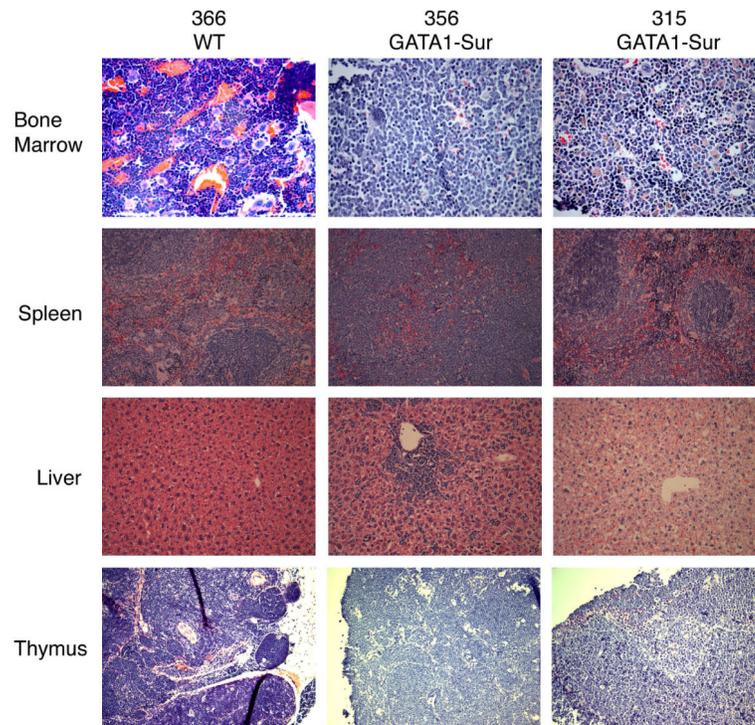


Figure 2. GATA1-Sur mice develop high-grade lymphoma upon ENU treatment

Hematoxylin and eosin stained sections of sternum bone marrow, spleen, liver and thymus show the presence of high-grade, aggressive lymphoma in one group of mice (e.g. mouse 356). A second set of animals (e.g. mouse 315) displayed lymphoma that was restricted to thymus and was accompanied by myeloproliferation in bone marrow and spleen.

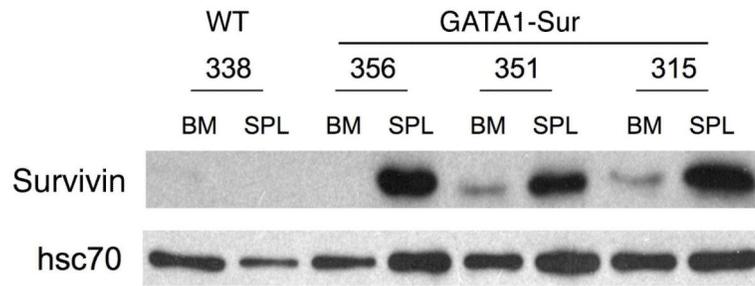


Figure 3. Survivin is highly expressed in spleens of diseased transgenic animals

The extent of survivin overexpression in tissues from diseased ENU-treated transgenic mice was analyzed by western blot analysis. Mouse 338, non-transgenic control; Mice 356, 351, and 315, diseased GATA1-Sur transgenics. Hsc70 was used as a loading control.

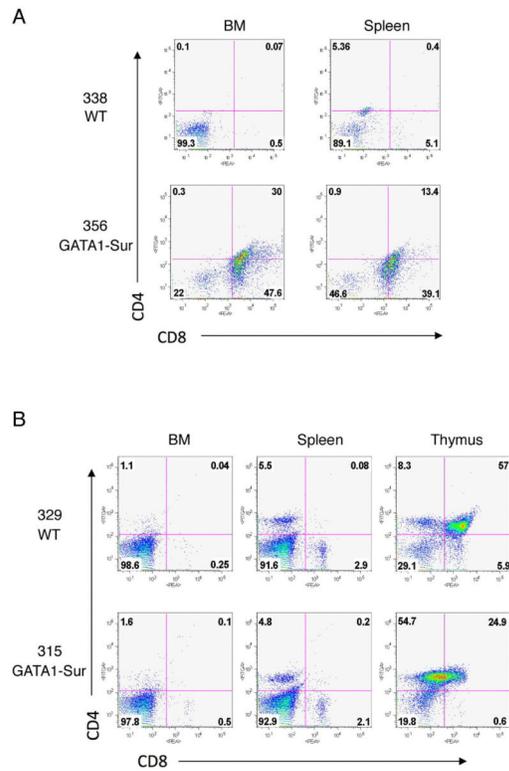


Figure 4. Flow cytometry reveals aberrant lymphoid proliferation in GATA1-Sur diseased mice
 Single cell suspensions of bone marrow, spleen and thymus of diseased GATA1-sur mice and control littermates were stained with anti-CD4 and anti-CD8 antibodies. (A) Animal 356 showed an aberrant expansion of T-cells in the bone marrow and spleen. (B) Animal 315 showed a localized expansion of abnormal T-cells in the thymus, but no evidence of lymphoma in the spleen or bone marrow.

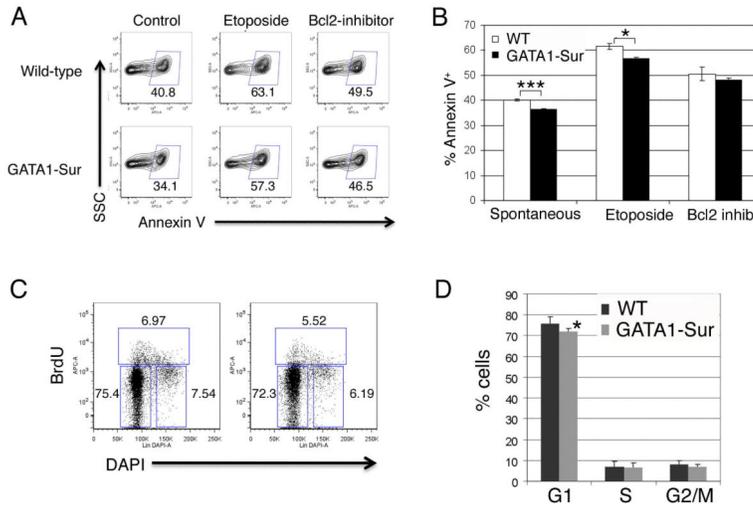


Figure 5. Splenocytes from GATA1-Sur transgenic mice show subtle differences in apoptosis and cell cycle progression compared to those of wild-type littermates

A, B) Splenocytes from GATA1-Sur mice or control littermates were cultured in vitro and treated with etoposide or Bcl2 inhibitor and then assayed for viability by annexin V staining. Representative flow plots (A) and means \pm standard deviations for three experiments (B) are shown. C, D) Splenocytes were cultured for three hours with BrdU and proliferation was assayed by flow cytometry. Representative flow plots (C) and means \pm standard deviations of three experiments (D) are shown. * $p < 0.05$, *** $p = 0.008$.

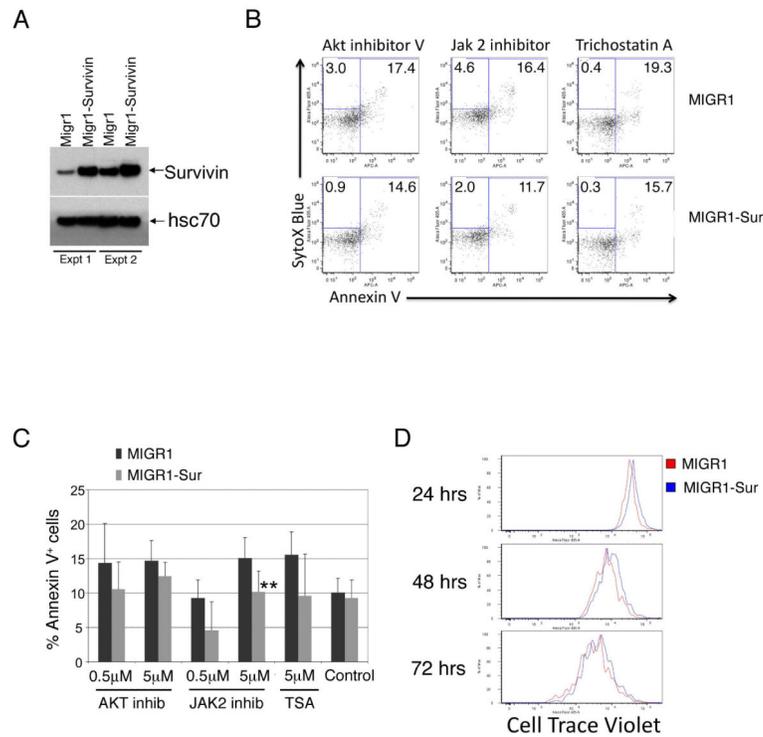


Figure 6. Overexpression of survivin protects primary T cells from death but does not affect proliferation

A) Primary murine T cells were activated ex vivo, transduced with control vector (MIGR1) or MIGR1-survivin and the extent of survivin expression was assessed by western blot. Hsc70 was used as a loading control. Proteins extracted from two separate experiments are shown. B, C) Aliquots of cells were treated with various inducers of cell death and assayed for viability by annexin V/sytoX blue staining. Representative flow plots (B) and means ± standard deviations for three experiments (C) are shown. D) Aliquots of cells were treated with cell trace violet and the cellular proliferation was evaluated by recording dye levels at 24, 48, and 72 hours. ** p=0.0005.