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Award Number: DAMD17-01-1-0167

TITLE: Are Diadenosine Polyphosphates and/or FHIT Involved in
Anoikis?

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REPORT DATE: June 2004

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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20040721 043

REPORT DOCUMENTATION PAGE*Form Approved*
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE June 2004	3. REPORT TYPE AND DATES COVERED Final (1 Jun 01 - 31 May 04)	
4. TITLE AND SUBTITLE Are Diadenosine Polyphosphates and/or FHIT Involved in Anoikis?			5. FUNDING NUMBERS DAMD17-01-1-0167	
6. AUTHOR(S) Steven M. Frisch, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The Burnham Institute La Jolla, California 92037 E-Mail: sfrisch@burnham.org			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) The FHIT (Fragile Histidine Triad) protein is an intriguing protein having certain properties of a tumor suppressor protein. The FHIT gene is altered in a large fraction of both sporadic and familial human breast cancers, genetically supporting its tumor gene status. Indeed, FHIT knockout mice have elevated frequency of various tumor types, consistent with this. The overexpression of the FHIT protein through recombinant adenovirus vector infection induces apoptosis and thus suppresses tumor growth, suggesting a more specific role for FHIT in apoptosis control. Yet, the biochemical activity and biologic function of FHIT protein are unknown at present, other than the fact that it binds and hydrolyzes the diadenosine polyphosphates Ap3A and Ap4A – molecules that accumulate in response to cellular stress. In this project we have focused on the potential role of FHIT in controlling one particularly cancer relevant apoptotic response – anoikis. Anoikis is the apoptotic response to cell-matrix detachment. It prevents mammary epithelial cells from colonizing in novel locations (i.e., metastasizing), thereby playing an important role in restricting mammary tumor progression that is often compromised in tumor cells. We have found that FHIT protein does not appear to play a role in anoikis, or, more generally, in apoptosis, prompting a re-examination of its actual cellular function. In the course of this project, however, we found a critical role for caspase-2 in anoikis.				
14. SUBJECT TERMS Anoikis/ApnA/Fhit/extracellular matrix/apoptosis			15. NUMBER OF PAGES 10	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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INTRODUCTION:

The FHIT (Fragile Histidine Triad) protein is an intriguing protein having certain properties of a tumor suppressor protein. The FHIT gene is altered in a large fraction of both sporadic and familial human breast cancers(1), genetically supporting its tumor gene status. Indeed, FHIT knockout mice have elevated frequency of various tumor types, consistent with this. The overexpression of the FHIT protein through recombinant adenovirus vector infection induces apoptosis and thus suppresses tumor growth (2),(3) suggesting a more specific role for FHIT in apoptosis control. Yet, the biochemical activity and biologic function of FHIT protein are unknown at present, other than the fact that it binds and hydrolyzes the diadenosine polyphosphates Ap3A and Ap4A – molecules that accumulate in response to cellular stress (1).

In this project we have focused on the potential role of FHIT in controlling one particularly cancer relevant apoptotic response – anoikis. Anoikis is the apoptotic response to cell-matrix detachment. It prevents mammary epithelial cells from colonizing in novel locations (i.e., metastasizing), thereby playing an important role in restricting mammary tumor progression that is often compromised in tumor cells (4).

BODY:

To summarize our results, we do not find evidence to support that FHIT has a role in anoikis, nor does overexpression of the protein after transfection (as opposed to adenovirus infection) affect apoptosis. However, in the course of these studies, we addressed the role of caspase-2 – which was reported to be activated by FHIT (5) – in anoikis, and indeed found an important role for this hitherto unsuspected caspase.

Accordingly, the focus of some of the specific aims had to be modified as explained below:

S.A. 1. Does cell adhesion regulate ApnA/Fhit in normal mammary epithelial cells?

1a-: Do ApnA levels change during anoikis?

Under specific aim 1c below, we show that FHIT is not apparently involved in anoikis. Therefore, measurements of Ap3A/Ap4A levels after detachment was not warranted. We have, however, assayed for the effect of cell detachment on FHIT protein levels after detachment, with results that will be presented below.

*1b: Is Fhit responsible for the changes in ApnA levels occurring during anoikis?
(no longer applicable, same reason as above).*

1c: Is Fhit involved in anoikis?

Results on this are presented below.

S.A. 2. How is ApnA/Fhit involved in anoikis in normal and transformed mammary epithelial cells?

2a. What is the epistatic relationship between ApnA/Fhit and anoikis-relevant oncogenes that are frequently activated in breast cancer?

This aim has been addressed within some of the experiments pertaining to specific aim 1c; see data below.

2b: Is ApnA/Fhit a core component of the apoptotic machinery?

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Because of the new role of caspase-2 in FHIT responses (5), we have focused on determining the role of caspase-2 in anoikis, in particular, because caspase-2 has recently become recognized as a possible alternative pathway to mitochondrial permeabilization (6) and is therefore, in a sense, a core component of the apoptotic machinery.

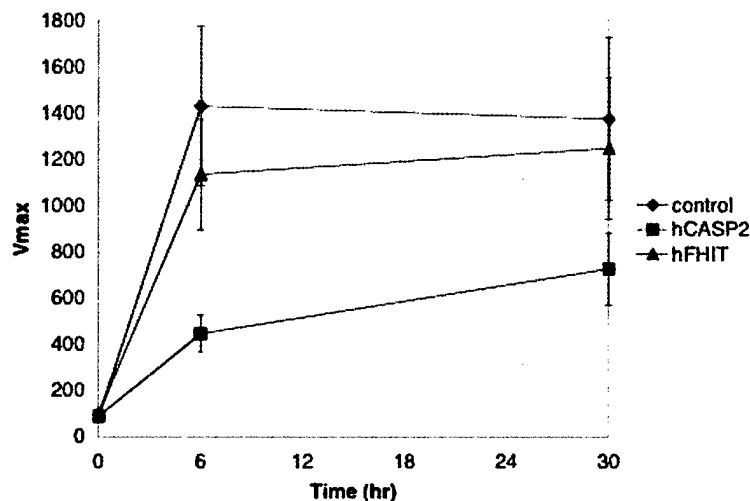
BODY:

We focused most of our efforts on determining the role of FHIT in anoikis, using primarily siRNA and overexpression strategies. In the original proposal, we were planning to use antisense to deplete FHIT protein, but this technology has largely been superseded since that time by the more reliable siRNA approach. We therefore designed duplex FHIT siRNA, transfected it into MCF10a mammary epithelial cells and analyzed equal amounts of protein for FHIT expression using a Western blot, with the result (figure 1), showing that this siRNA was highly effective in reducing FHIT expression:

FHIT siRNA: - +

Figure 1. FHIT siRNA reduces FHIT protein levels effectively. Western blot was performed on MCF10a cells that had been transfected with FHIT siRNA, using BD anti-FHIT antibody.

We then assayed siRNA-treated MCF10a cells for anoikis. Previously, we had reported that omission of the growth factors insulin and EGF –normal components of the growth medium of MCF10a cells – during suspension promoted anoikis, so we had used this omission strategy to increase the sensitivity of the assay. More recently, however, we obtained evidence showing that EGF/insulin omission contributed significantly to apoptosis even in confluent attached MCF10a cells (data not shown). We therefore assayed for anoikis here in the presence of these factors to avoid possible artifacts from this source, using DEVD-AFC cleavage as a fluorimetric readout of caspase activation in lysates derived from cells suspended for the indicated periods of time. We also compared the effect of a well-characterized caspase-2 siRNA (7) that is known to be effective.



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Figure 2. FHIT siRNA does not protect against anoikis, but caspase-2 siRNA does. DEVD-AFC cleavage as a fluorimetric readout of caspase activation in lysates derived from cells transfected with siRNAs and then suspended for the indicated periods of time.

These experiments (figure 2) clearly indicate that FHIT siRNA did not have a significant effect on anoikis in MCF10a cells. By contrast, caspase-2 siRNA protected very efficiently, a novel result that is discussed further below.

FHIT has been reported to induce apoptosis and suppress tumor growth after adenoviral FHIT transduction into FHIT-deficient tumor cells. We therefore examined and compared the potential pro-apoptotic effect of FHIT in normal (FHIT-wild-type) and FHIT-deficient tumor cells to determine whether there was a differential pro-apoptotic effect in these contexts. We subcloned full-length FHIT cDNA into pcDNA3.1myhis, and showed that it was indeed expressed after transfection into MDCK or src/MDCK cells, by probing for the 6Xhis tag, showing high level expression (figure 3).

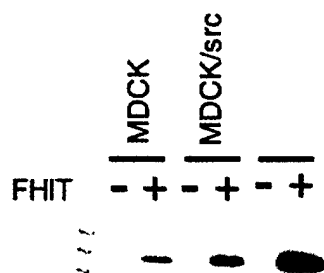


Figure 3. High level expression of FHIT, revealed after transient transfection into MDCK or MDCK/src cells and probed with anti-tetra-his antibody.

We then cotransfected a GFP vector with cotransfected FHIT or empty pcDNA3.1 vector into MDCK, src-transformed MDCK or the FHIT-null tumor cell line MDA-MB436, and scored for GFP positive cells surviving after one or two days (figure 4).

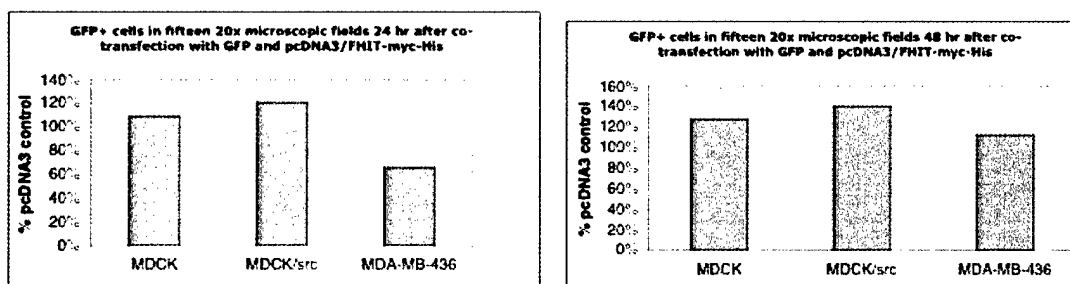


Figure 4. FHIT overexpression by transfection does not induce apoptosis. The indicated cell lines were cotransfected with EGFP-C3 plus FHIT or empty pcDNA3.1 vector and the number of surviving GFP-positive cells was scored. The y-axis reflects the percent change of GFP-positive, FHIT-positive cells compared to GFP-positive, FHIT-negative cells that survived after transfection.

These results clearly indicate that FHIT did not significantly induce apoptosis in normal epithelial cells (MDCK), transformed FHIT-wild-type cells (MDCK/src) or FHIT-null

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tumor cells. This is in contrast with previous reports in which adenovirus FHIT vectors were shown to induce apoptosis, most likely reflecting the much higher expression levels (2-5% total cell protein in some cases) that adenovirus vectors produce. This result, and the siRNA results above, cast serious doubt on the proposition that FHIT protein normally functions to regulate apoptosis. This negative result was further confirmed by a similar experiment in which MDCK, MDA-MB436 or another FHIT-negative cell line SW480 were cotransfected with GFP with or without FHIT and apoptosis was scored by analyzing the GFP-positive (gated) population for uptake of propidium iodide (figure 5):

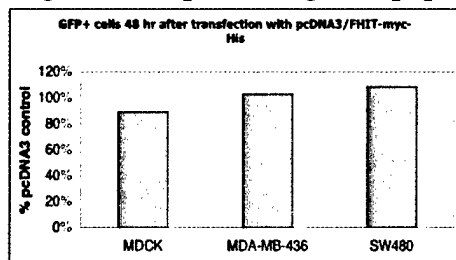


Figure 5. FHIT overexpression does not induce apoptosis (FACS). A similar experiment to figure 4 in which MDCK, MDA-MB436 or another FHIT-negative cell line SW480 were cotransfected with GFP with or without FHIT and apoptosis was scored by analyzing the GFP-positive (gated) population for uptake of propidium iodide.

We then tested the ability of overexpressed FHIT to sensitize normal epithelial cells to anoikis. MCF10a or MDCK cells were cotransfected with a GFP vector plus FHIT or empty vector. After growth to confluence, cells were suspended for 6 hours, fixed, stained with propidium iodide and the GFP-positive cell population was analyzed for anoikis (PI-positivity) by FACS, with the results depicted below (figure 6).

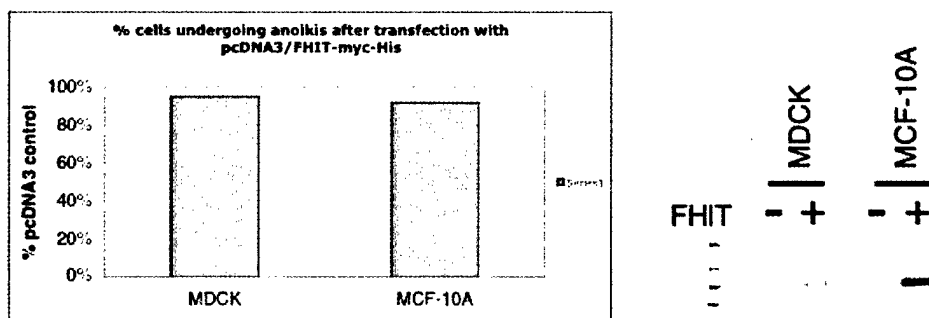


Figure 6. Overexpressed FHIT does not sensitize epithelial cells to anoikis. MCF10a or MDCK cells were cotransfected with a GFP vector plus FHIT or empty vector. After growth to confluence, cells were suspended for 6 hours, fixed, stained with propidium iodide and the GFP-positive cell population was analyzed for anoikis (PI-positivity) by FACS, with the results depicted below (the y-axis reflects the percent change of anoikis in FHIT-positive cells compared to FHIT-negative cells).

Clearly, FHIT expression did not sensitize epithelial cells to anoikis.

Consistent with this, there was also no significant change in the level of FHIT protein during the time course of anoikis, as shown by the Western blot below (figure 7).

0 1 6 24 hr suspension
 - - - - FHIT

Figure 7. FHIT protein levels do not change in response to cell detachment. Western blot on detached MCF10a cells using anti-FHIT mAb (BD).

This result suggests that, if there is a change in the level of Ap3A/Ap4A during the course of anoikis, it is probably not effected by a change in FHIT levels, and, in any event, FHIT activity does not have an effect on the magnitude of anoikis.

Finally, motivated by a report that caspase-2 is activated during the course of FHIT-induced apoptosis, we examined the role of caspase-2 in anoikis, with the following results.

1. Caspase-2-like activity is activated early after detachment of epithelial cells (MCF10a) from matrix. This was determined by assaying cell lysates at increasing times after detachment for caspase-3/7-like (DEVD-AFC cleaving) or caspase-2-like (VDVAD-AFC cleaving) activities (figure 8):

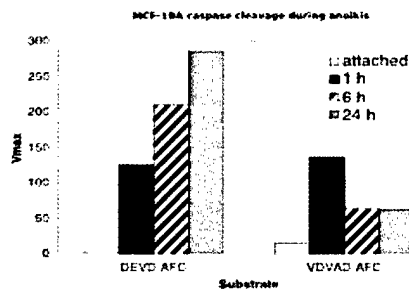


Figure 8. Caspase-2 activity appears early after detachment of cells from matrix. Caspase-2-like activity is activated early after detachment of epithelial cells (MCF10a) from matrix. This was determined by assaying cell lysates at increasing times after detachment for caspase-3/7-like (DEVD-AFC cleaving) or caspase-2-like (VDVAD-AFC cleaving) activities.

2. Caspase-2 activation in the early phase of anoikis could also be detected (figure 9) in terms of cleavage of the proenzyme (Western blot, left) or affinity labeling in vivo with bio-VAD-fmk followed by streptavidin precipitation and Western blotting with anti-caspase-2 monoclonal, revealing only the active enzyme (right):

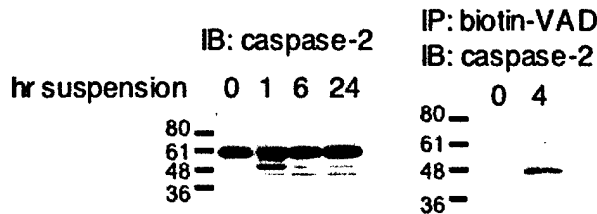


Figure 9. Caspase-2 activation seen by two other methods: caspase-2 proenzyme processing (left) and in vivo affinity labeling/Western blotting (right).

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3. Caspase-2 siRNA protected cells efficiently against anoikis, as measured by total caspase activation –see figure 1.

4. Caspase-2 siRNA protected cells efficiently against anoikis, as measured by a “Live-Dead” assay (i.e., conversion of calceinAM to a green fluorescent product = live; uptake of ethidium bromide = Dead, both measured simultaneously on a plate reader):

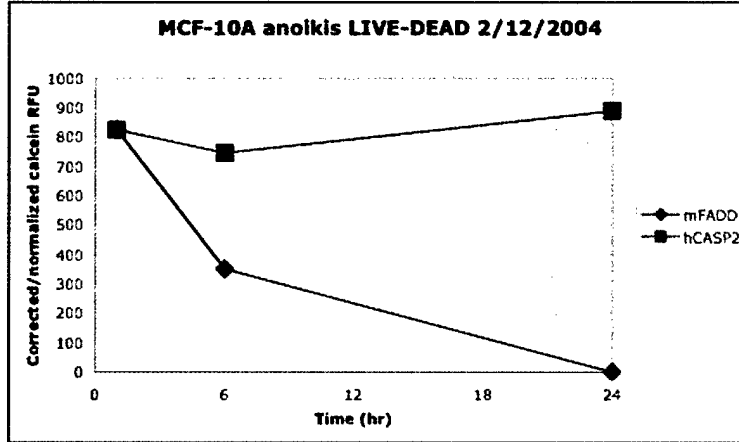


Figure 10. Caspase-2 siRNA protects against anoikis-LIVE-DEAD assay. The LIVE cell signal is shown as a function of time in suspension. MFADD siRNA was used as a negative control, because it is a mouse sequence used on human (MCF10a) cells.

5. The caspase-2-selective peptide inhibitor z-VDVAD-fmk protected cells efficiently against anoikis, as measured by PARP cleavage:

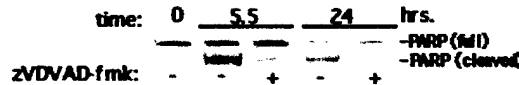


Figure 11. Caspase-2 inhibitor blocks anoikis. MCF10a cells in suspension were treated with 50 micromolar z-VDVAD-fmk and assayed for PARP cleavage by Western blotting.

KEY RESEARCH ACCOMPLISHMENTS:

--FHIT does not appear to be a regulator of apoptosis/anoikis, using a variety of criteria. This suggests that FHIT regulates other aspects of cell behavior, awaiting identification.

--FHIT levels are not regulated by cell-matrix adhesion.

--Caspase-2 plays a critical role in the anoikis response.

REPORTABLE OUTCOMES:

None.

CONCLUSIONS:

The results strongly suggest that research on the genetically FHIT direction should probably be re-focused on aspects of cell behavior such as cell-cycle control, motility/invasion, angiogenesis, rather than on apoptosis. This is an important re-definition of priorities in the FHIT field.

Secondly, the results suggest that caspase-2 should be re-examined in terms of anoikis, and, by inference, tumor progression. More effort should be invested into understanding its mechanism of activation, regulation by cell adhesion and possible substrates.

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