

UNCLASSIFIED

AD NUMBER
ADB266144
NEW LIMITATION CHANGE
TO Approved for public release, distribution unlimited
FROM Distribution authorized to U.S. Gov't. agencies only; Proprietary Info.; Jul 2000. Other requests shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott St., Fort Detrick, MD 21702-5012.
AUTHORITY
USAMRMC ltr, 5 Mar 2002

THIS PAGE IS UNCLASSIFIED

AD _____

Award Number: DAMD17-98-1-8078

TITLE: Functional Significance of Transcriptional Regulation by
VEGF Receptor Tyrosine Kinases

PRINCIPAL INVESTIGATOR: Adrienne Wong
Kevin Peters, M.D.

CONTRACTING ORGANIZATION: Duke University Medical Center
Durham, North Carolina 27710

REPORT DATE: July 2000

TYPE OF REPORT: Annual Summary

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

DISTRIBUTION STATEMENT: Distribution authorized to U.S. Government agencies only (proprietary information, Jul 00). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20010509 110

NOTICE

USING GOVERNMENT DRAWINGS, SPECIFICATIONS, OR OTHER DATA INCLUDED IN THIS DOCUMENT FOR ANY PURPOSE OTHER THAN GOVERNMENT PROCUREMENT DOES NOT IN ANY WAY OBLIGATE THE U.S. GOVERNMENT. THE FACT THAT THE GOVERNMENT FORMULATED OR SUPPLIED THE DRAWINGS, SPECIFICATIONS, OR OTHER DATA DOES NOT LICENSE THE HOLDER OR ANY OTHER PERSON OR CORPORATION; OR CONVEY ANY RIGHTS OR PERMISSION TO MANUFACTURE, USE, OR SELL ANY PATENTED INVENTION THAT MAY RELATE TO THEM.

LIMITED RIGHTS LEGEND

Award Number: DAMD17-98-1-8078
Organization: Duke University Medical Center
Location of Limited Rights Data (Pages):

Those portions of the technical data contained in this report marked as limited rights data shall not, without the written permission of the above contractor, be (a) released or disclosed outside the government, (b) used by the Government for manufacture or, in the case of computer software documentation, for preparing the same or similar computer software, or (c) used by a party other than the Government, except that the Government may release or disclose technical data to persons outside the Government, or permit the use of technical data by such persons, if (i) such release, disclosure, or use is necessary for emergency repair or overhaul or (ii) is a release or disclosure of technical data (other than detailed manufacturing or process data) to, or use of such data by, a foreign government that is in the interest of the Government and is required for evaluational or informational purposes, provided in either case that such release, disclosure or use is made subject to a prohibition that the person to whom the data is released or disclosed may not further use, release or disclose such data, and the contractor or subcontractor or subcontractor asserting the restriction is notified of such release, disclosure or use. This legend, together with the indications of the portions of this data which are subject to such limitations, shall be included on any reproduction hereof which includes any part of the portions subject to such limitations.

THIS TECHNICAL REPORT HAS BEEN REVIEWED AND IS APPROVED FOR PUBLICATION.



- 04/05/01

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 July 2000	3. REPORT TYPE AND DATES COVERED Annual Summary (1 Jul 99 - 30 Jun 00)	
4. TITLE AND SUBTITLE Functional Significance of Transcriptional Regulation by VEGF Receptor Tyrosine Kinases			5. FUNDING NUMBERS DAMD17-98-1-8078	
6. AUTHOR(S) Adrienne Wong Kevin Peters, M.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Duke University Medical Center Durham, North Carolina 27710 E-MAIL: alw5@acpub.duke.edu			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 This report contains colored photos				
12a. DISTRIBUTION / AVAILABILITY STATEMENT DISTRIBUTION STATEMENT: Distribution authorized to U.S. Government agencies only (proprietary information, Jul 00). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 Words) Angiogenesis plays a fundamental role in solid tumor growth and metastasis. The vasculature is for the most part quiescent in the adult, so it follows that anti-angiogenic therapies are an attractive method of targeting tumorigenesis. Thus, the purpose of this study was to study the basic mechanisms behind vascular development with the goal of discovering novel proteins involved in angiogenic signal transduction pathways. This project both informs the understanding of angiogenesis and has the potential benefit of identifying novel, specific targets for anti-angiogenic therapy. Two families of endothelial receptor tyrosine kinases play an essential role in angiogenesis: VEGF and Tie. In order to find new members of this signaling pathway, we performed a yeast two-hybrid screen of a human fetal heart library using the VEGFR1 kinase domain as bait. We discovered that VEGFR1 associated with SOCS2 in a specific, kinase-dependent manner. SOCS2 is a member of the SOCS family of cytoplasmic signaling proteins that share homology in their C-terminal SOCS-box. Interestingly, other members of the family demonstrate a negative regulatory effect on cytokine signaling. Thus, we hypothesized that SOCS2 has a role on VEGF growth factor signaling through the VEGFR1 receptor.				
14. SUBJECT TERMS Breast Cancer, Angiogenesis, Receptor Tyrosine Kinases, VEGF			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

Table of Contents

Cover		
SF 298	..	2
Foreword	.	3
Table of Contents	.	4
Introduction	5
Body	.	6-8
Key Research Accomplishments	.	9
Reportable Outcomes	.	9
Conclusions	..	9
References		n/a
Appendices		

Introduction

Angiogenesis plays a fundamental role in solid tumor growth and metastasis. The vasculature is for the most part quiescent in the adult, so it follows that anti-angiogenic therapies are an attractive method of targeting tumorigenesis. Thus, the purpose of this study was to study the basic mechanisms behind vascular development with the goal of discovering novel proteins involved in angiogenic signal transduction pathways. This project both informs the understanding of angiogenesis and has the potential benefit of identifying novel, specific targets for anti-angiogenic therapy.

Two families of endothelial receptor tyrosine kinases play an essential role in angiogenesis: VEGF and Tie. In order to find new members of this signaling pathway, we performed a yeast two-hybrid screen of a human fetal heart library using the VEGFR1 kinase domain as bait. We discovered that VEGFR1 associated with SOCS2 in a specific, kinase-dependent manner. SOCS2 is a member of the SOCS family of cytoplasmic signaling proteins that share homology in their C-terminal "SOCS-box". Interestingly, other members of the family demonstrate a negative regulatory effect on cytokine signaling. Thus, we hypothesized that SOCS2 has a role on VEGF growth factor signaling through the VEGFR1 receptor.

Body

This work differs from that originally outlined in the approved statement of work. The cDNA RDA screen proposed in my original statement was performed to completion (see annual report, 1999). Unfortunately this screen as performed did not yield any novel proteins which were transcriptionally regulated by VEGF, making impossible to go further with that project. Reasons for the failure of this screen could include: 1. looking at the wrong timepoints for transcriptional regulation, 2. using a cell line that for whatever reason did not induce protein transcription in the way that an endothelial cell in vivo would, or indeed 3. the lack of a novel transcriptionally regulated protein to be found. Therefore, the following summary focuses on our study of an earlier stage of VEGF-induced angiogenesis, signal transduction. The signal transduction pathways of angiogenesis have not been completely elucidated and are equally important to the overall understanding of tumor angiogenesis.

The relationship between angiogenesis and solid tumor progression has been well established. Therefore, an understanding of the basic mechanisms of angiogenesis benefits not only the vernacular but may lead to novel cancer-targeting therapeutics.

Receptor Tyrosine Kinases (RTKs) and their cognate ligands play important roles in vascular growth and development . We performed a screen for novel members of the VEGF signaling pathway, to answer two important questions:

- What signal transduction events occur post growth-factor stimulation of endothelial RTKs?
- How do they affect specific aspects of the growth and maintenance of the vascular network?

The Flt-1 kinase domain was used as bait in a yeast-two hybrid screen of a human fetal heart library (in collaboration with M. Blonar, Bristol-Myers Squibb). This screen demonstrated a novel association between Flt-1 and SOCS2, a SH2-domain containing cytoplasmic protein.

The SOCS family, so named for their function as “Suppressors Of Cytokine Signaling” display a homology via their C-terminal “SOCS box”. The SOCS box has a characteristic N-terminal BC box and C-terminal L/P rich sequence, and its function has been under debate: it is either a protein stabilizer or a targeting protein for protein degradation. Several members of this family have been shown to function as negative regulators of signal transduction. They affect JAK/STAT signaling via the SH2 domain and an N terminal kinase inhibitory region. Thus, our hypothesis is that SOCS2 modulates VEGF-induced angiogenesis by binding to the RTK via its SH2 domain.

Further bait testing demonstrated a kinase- or phosphotyrosine-dependent association between Flt-1, Flk-1 and Tie-2 and SOCS2 (Fig. 1). SOCS2 was tested for association with baits consisting of the kinase domains of the four main endothelial RTKs. KR denotes a point mutation in the ATP-binding site rendering the bait protein kinase-deficient.

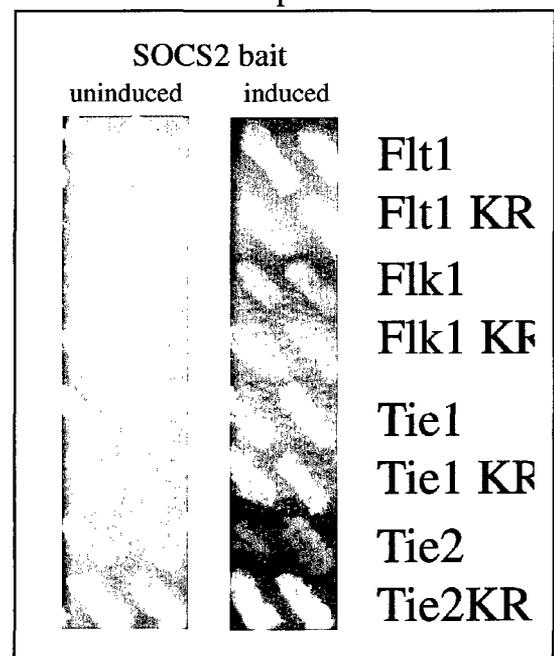


Figure 1

Using a GST-pulldown assay, Flt-1 kinase and Flk-1 kinase both associate with the SH2 domain of SOCS2 in a kinase-dependent manner (Figs. 2-4). In figure 3, equal amounts of wild type (wt) and kinase-inactive (KR) GST-Flt-1 kinase were bound to beads [GST, PY99 blots]. Flag-SOCS2 bound exclusively to the wt and not the KR. Flag-SOCS2RK, with a point mutant in its SH2 domain, did not bind to either wt or KR GST-Flt-1. [Flag blot] The association of PLC γ and Grb2 with wt GST-Flt-1 was not affected by overexpression of SOCS2 [PLC γ , Grb2 blots]. In figure 4, the same is seen with GST-Flk-1 kinase.

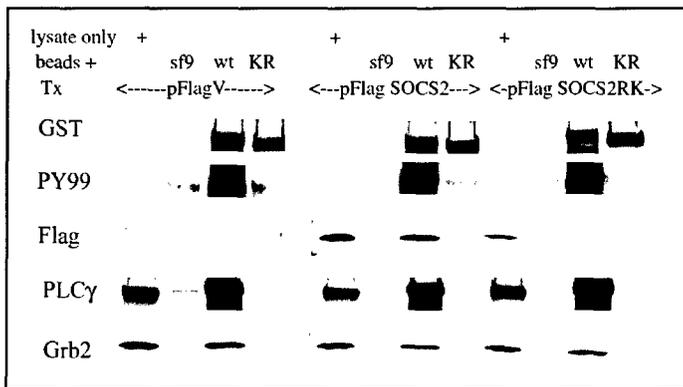
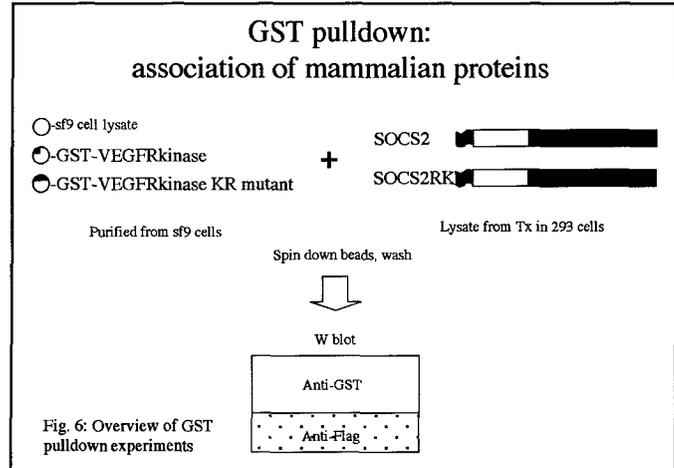


Figure 3: GST-Flt kinase association w/ SOCS2

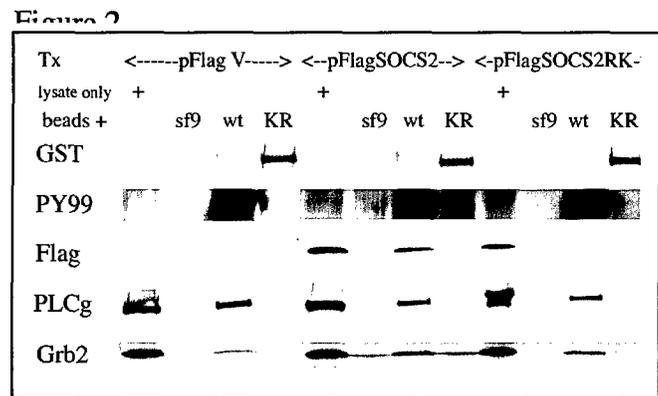
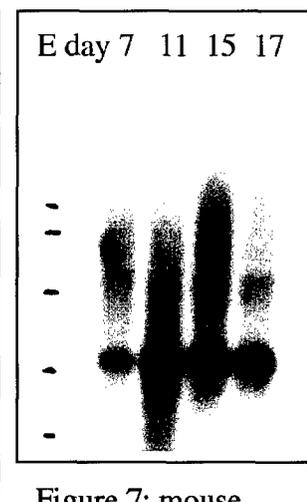
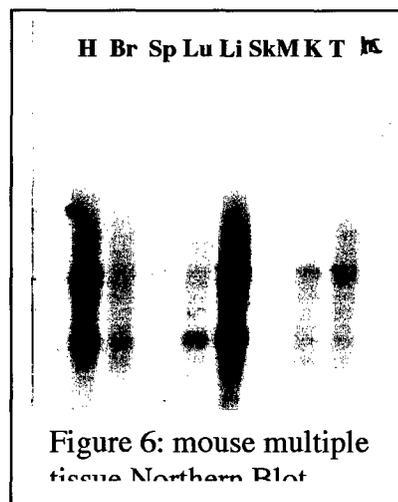
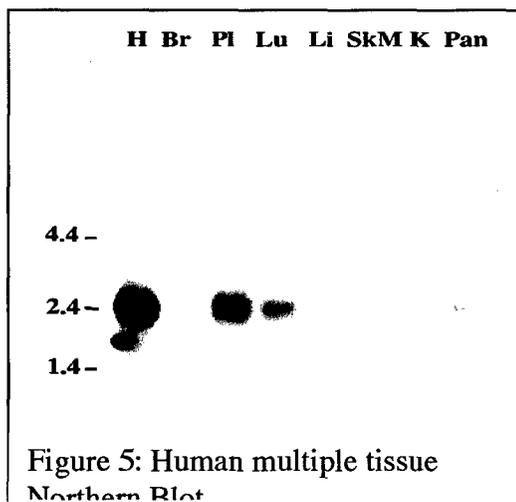


Figure 4: GST-Flk kinase association w/ SOCS2

Northern blotting demonstrated SOCS2 expression in highly vascularized adult tissues and at various developmental stages (Fig. 5-7). Northern blots were probed with either murine or human SOCS2 probe. H=heart, Br=brain, Pl=placenta, Lu=lung, Li=Liver, SkM=skeletal muscle, K=kidney, Pan=pancreas, Sp=spleen, T=testes. Mouse developmental blot contains poly A RNA from embryonic day 7, 11, 15, and 17.



Whole-mount *in situ* hybridization of mouse lung reveals SOCS2 mRNA expression in the mesenchyme surrounding developing lung buds at day 12.5, consistent with an area of developing vasculature (Fig. 8)

Immunohistochemistry demonstrates SOCS2 protein expression in the endothelium of placental blood vessels, a site of active angiogenesis (Fig. 9). This polyclonal antibody against human SOCS2 was generated by our laboratory and purified against a His-SOCS2 affinity column.

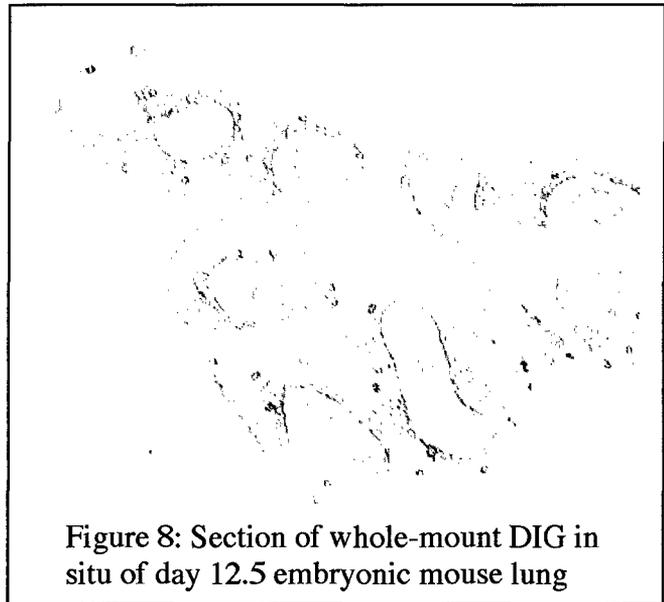


Figure 8: Section of whole-mount DIG in situ of day 12.5 embryonic mouse lung

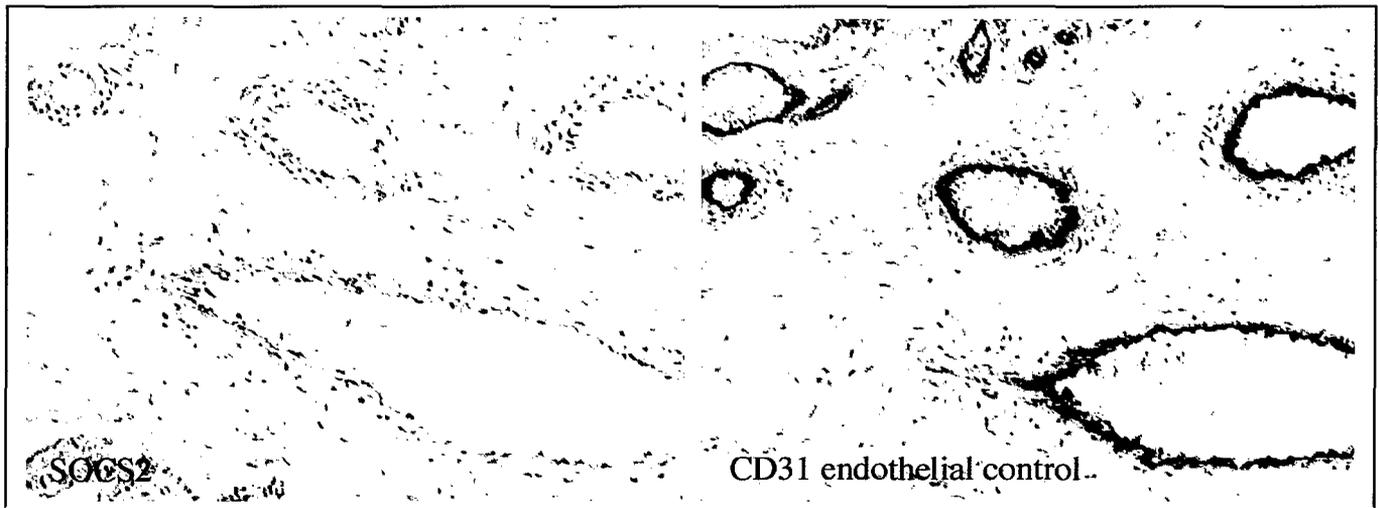


Figure 9: Immunohistochemical staining of SOCS2 in placental blood vessels

In summary,

1. The association of VEGFR1 and VEGFR2 with SOCS2 has been demonstrated in both the yeast two-hybrid system and a GST-pulldown system.
2. This association is dependent upon the presence of both a functional kinase domain on the receptor and an intact SH2 domain in SOCS2.
3. Expression studies have detected SOCS2 mRNA in highly vascularized tissues and SOCS2 protein in the endothelium of the placenta, adding weight to the hypothesis that the *in vitro* association between SOCS2 and VEGF receptors occurs *in vivo*.

Future directions include:

1. Determine how VEGF stimulation regulates SOCS2 association with VEGF receptors in endothelial cells.
2. Determine function of SOCS2 interaction with VEGFR in angiogenesis

Key Research Accomplishments:

- Demonstrated via the yeast two-hybrid system a novel association between VEGFR1 and VEGFR2 with SOCS2.
- Demonstrated via a GST-pulldown system the novel association between VEGFR1 and VEGFR2 with SOCS2.
- Demonstrated that this association is dependent upon the presence of both a functional kinase domain on the receptor and an intact SH2 domain in SOCS2.
- Demonstrated that SOCS2 mRNA is expressed in highly vascularized tissues, and SOCS2 protein is expressed in the endothelium of the placenta.

Reportable Outcomes:

Abstracts and Presentations:

Poster Presentation, "Role of SOCS2 in VEGF and Tie-mediated angiogenesis",
Era of Hope Meeting, AMRMC Breast Cancer Research Program, June 2000.

Poster Presentation, "Novel Association between Flt-1, Flk-1, Tie2 and SOCS2 in the yeast two-hybrid system", **American Society for Cell Biology Annual Meeting, December 1999**

Development of a replication-deficient Adenovirus which overexpresses SOCS2 wild-type protein.

Conclusions:

We have clearly demonstrated a novel interaction between the VEGFR1 and VEGFR2 receptors with the SH2-domain containing protein SOCS2. This interaction occurs in both the yeast two hybrid system and the GST-pulldown system with mammalian proteins, and is dependent upon both a functional kinase domain and the presence of an intact SH2 domain. SOCS2 is present in the endothelium, so it would follow that this interaction has a functional significance *in vivo*. Further experiments will attempt to elucidate the function of this interaction *in vivo*.



7

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF.

MCMR-RMI-S (70-1y)

5 Mar 02

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statements

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for grants. Request the limited distribution statements for Accession Documents listed at enclosure be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

A handwritten signature in black ink, appearing to read "Phyllis M. Rinehart", is written over the typed name and title.

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

Encl

DISTRIBUTION TO BE CHANGED TO UNLIMITED,
APPROVED FOR PUBLIC RELEASE

ACCESSION DOCUMENT NUMBERS

ADB267943	ADB267947
ADB257308	ADB268439
ADB233733	ADB242952
ADB263445	ADB248375
ADB258807	ADB240661
ADB257354	ADB271166
ADB240907	ADB258706
ADB270764	ADB244250
ADB241926	ADB258773
ADB246527	ADB254490
ADB268422	ADB268189
ADB252915	ADB270793
ADB258877	ADB266144
ADB268117	ADB236672
ADB267884	ADB259031
ADB254260	ADB270765
ADB268296	ADB270785
ADB258930	ADB268113
ADB271098	ADB270791